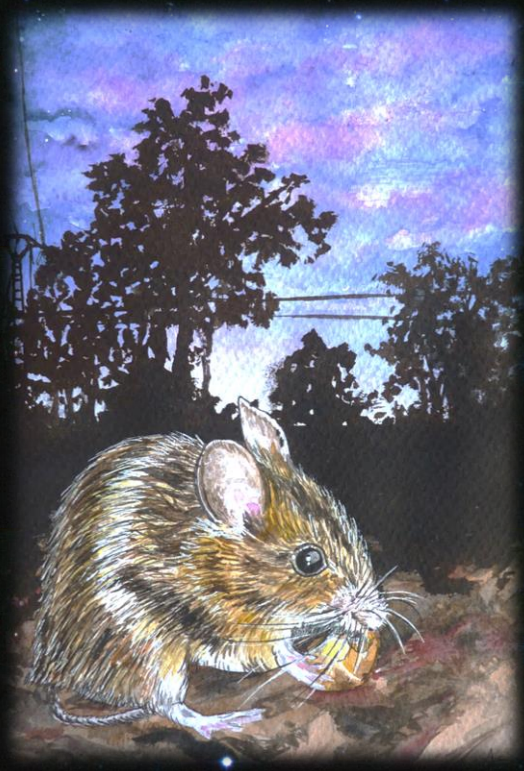
Efecto de Diferentes Factores Ambientales, del Riesgo de Depredación y de los Niveles de Testosterona sobre la Modulación de las Respuestas Comportamental y de Estrés Fisiológico en el Ratón de Campo (*Apodemus sylvaticus*)

Tesis Doctoral



Beatriz Sánchez González

Madrid 2017

UNIVERSIDAD AUTÓNOMA DE MADRID
Facultad de Ciencias
Departamento de Biología

Imágenes de portada y contraportada: ratón de campo *Apodemus sylvaticus* y zorro rojo *Vulpes vulpes*
Autora: Ana Sagredo Ardisana

Ilustraciones de los capítulos realizadas por José Luis Sánchez Calleja.

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Memoria presentada por **Beatriz Sánchez González** para optar al grado de Doctor en Ciencias Biológicas por la Universidad Autónoma de Madrid

Bajo la dirección de la **Dra. Isabel Barja Núñez**, Profesora del Departamento de Biología de la Universidad Autónoma de Madrid

El doctorando

Vº Bº de la Directora

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Dra. Isabel Barja Núñez

Madrid, Noviembre 2017

A todos los que confiasteis en mí
A mi pequeña gran familia

AGRADECIMIENTOS

Me gustaría empezar dando las gracias a la persona que ha hecho posible que hoy esté escribiendo mi tesis doctoral, la Dra. Isabel Barja Núñez. Gracias Isa por todo tu apoyo durante estos cuatro años, dedicándome tu tiempo e infinita paciencia para enseñarme, aconsejarme y animarme a hacer las cosas bien. Empezamos en el Máster cuando apenas había visto un ratón en mi vida y me has acompañado en todo este largo proceso confiando en mí y transmitiéndome tus valiosos conocimientos sobre el mundo de la investigación. Muchas gracias por estar siempre dispuesta a pasar tardes eternas en tu despacho planificando nuestros experimentos, y digo nuestros porque sin ti no hubiesen sido posible, y por sacar tiempo de donde no lo tenías para echar un vistazo a la versión buena_buenísima de mis trabajos. Has estado siempre con una palabra de ánimo y me has comprendido en mi indecisión. Espero poder seguir a tu lado aprendiendo y compartiendo todos los proyectos que puedan venir tanto de campo, a los que encantada madrugaré para ver a mi querido ratón de campo, como docentes, donde sabes que tienes una "aliada" para innovar. Gracias por formar parte de este camino.

Mi paso por el laboratorio no hubiese sido lo mismo sin ti, Dr. Álvaro Navarro Castilla. Desde el principio me has apoyado en todo lo que he necesitado, acompañándome al campo a horas intempestivas, siendo un magnífico chofer y sobre todo dándome los ánimos que a veces me faltaban. Gracias por compartir conmigo tanto y por tener siempre un hueco en tu agenda roja para revisarme con atención los borradores que te mandaba. Compañero de Congresos, ojalá el siguiente sea en una isla paradisiaca y vayamos, eso sí, en avión. Eres una gran persona y un increíble investigador, no puedo

estar más feliz de que formes parte de esta tesis y de mi vida. Gracias Alvarito.

También me gustaría agradecerle todo el apoyo recibido por parte de la Universidad Autónoma de Madrid, ofreciéndome siempre los medios necesarios para que esta tesis se pudiese realizar con éxito. En concreto a la Unidad de Zoología. Todos habéis participado en mi formación como docente, investigadora y persona, haciéndome sentir como en casa mientras aprendía lo que era el día-día de una gran Universidad. En especial, gracias al Dr. Javier de Miguel por las conversaciones zoológicas en las horas de autobús hacia las Lagunas de La Mancha, y por estar siempre dispuesto a ayudarme en todo lo que he necesitado. Espero poder disfrutar de tus valiosos conocimientos también en este final de etapa juntos. Al Dr. Juan Moreira por tus consejos sobre cómo publicar un Nature, que no olvidaré por si algún día tengo que ponerlos en práctica. Finalmente también me gustaría dar las gracias al Dr. Eduardo López por haber revisado en numerosas ocasiones este trabajo como tribunal de los planes de investigación, y a Mª Carmen Hernández, por acompañarme en esta última fase, te deseo mucha suerte en tu maravillosa aventura.

Al Centro de Naturaleza Open Cañada Real (Peralejo, Madrid), sobre todo a José España por proporcionarnos el material necesario para la realización de esta tesis, estando siempre dispuesto a colaborar.

A la Xunta de Galicia por facilitarme los permisos necesarios para llevar a cabo los experimentos y poner a mi disposición un alojamiento en el Parque Natural Montes do Invernadeiro (Ourense). A todos los agentes forestales por su buena atención durante nuestra estancia allí.

Son muchas las personas que han formado parte de este proyecto y a todas os doy las gracias. A los del Máster de Biodiversidad Celia, Calle y Esther (gracias sepios por animarme y aguantar mis confesiones) y a los que nos dejaron unirnos como uno más a Austria y Viena. Gracias a Esther y Cape por acompañarme a los muestreos (¡incluso cuando llovía!) y hacerme la espera mucho más amena, ya sabemos lo que pueden tardar los zorros en hacer ciertas cosas. Muchísimas gracias a la artistaza Ana por saber plasmar tan bien lo que este trabajo significa para mí en una sola imagen.

Por último, millones de gracias a las personas a las que va dedicado todo este trabajo, mis padres Chus y José Luis. Gracias y perdón por todo lo que habéis tenido que hacer por mí. Muchas gracias por ser mis secretarios en las madrugadas heladoras, por buscar soluciones a todos los problemas que se me planteaban, por coger el primer tren a Madrid en cuanto decía "mañana no tengo gente que me acompañe", gracias porque si no es por vosotros me hubiese perdido en este camino. No me olvido de ti, Mario, que aunque sé que no te hacía mucha gracia eso de madrugar para encima pasar frío (por si hay algo peor todavía que madrugar) siempre has estado dispuesto a acompañarme gastando tiempo de tus vacaciones, gasolina, ruedas y mucha paciencia.

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RESUMEN

El ambiente en el que viven los seres vivos varía constantemente. Por esta razón, animales como los micromamíferos, han desarrollado un conjunto de adaptaciones morfológicas, comportamentales y fisiológicas para hacer frente a la diversidad de amenazas y riesgos que les rodean. Así, desencadenar la respuesta adecuada para cada situación supone una ventaja vital a tener en cuenta, ya que aumenta las probabilidades de sobrevivir a corto (ej. evitar el encuentro con el depredador) y largo plazo (ej. evitar gastos energéticos costosos). Entre los numerosos peligros destacan la depredación por afectar directamente a la supervivencia de las especies presa y diversos factores ambientales que influyen en la calidad del hábitat y, por tanto, en los recursos que éste les ofrece. De este modo, en la presente tesis doctoral se estudiaron las respuestas comportamental y fisiológica de estrés del ratón de campo (*Apodemus sylvaticus* Linnaeus, 1758) ante diferentes grados de riesgo de depredación por zorro rojo (*Vulpes vulpes* Linnaeus, 1758) y factores ambientales, analizando la influencia de los factores individuales como sexo, estado reproductor y experiencia adquirida, en ambas respuestas. Además, también se analizó como varía la ingesta de alimento del ratón de campo en función de los niveles de metabolitos de testosterona fecal. La respuesta comportamental del ratón de campo se estudió mediante trampeos de vivo en dos áreas de estudio, el Monte de Valdelatas (Madrid, España) y el Parque Natural Os Montes do Invernadeiro (Galicia, España). La respuesta fisiológica de estrés fue examinada siguiendo una metodología no invasiva mediante la recolección de las heces frescas de los individuos capturados y la posterior cuantificación de los metabolitos de corticosterona fecal (MCF) y

testosterona fecal (MTF) mediante un enzimo inmunoensayo. Los resultados obtenidos mostraron que el ratón de campo es capaz de evaluar el grado de riesgo de depredación en función de diferentes concentraciones de olor fecal de depredador. A este respecto, los individuos reproductores fueron los que más evitaron la entrada en las trampas donde el riesgo de depredación percibido era máximo. Así mismo, la respuesta fisiológica de estrés aumentó conforme aumentaba el riesgo de depredación percibido por la presa, siendo los individuos reproductores los que presentaron niveles mayores de MCF. En cuanto a la ingesta de alimento, ésta aumentó significativamente con el olor de depredador, incrementándose en la parcela de máxima concentración. Las hembras y los individuos no reproductores fueron los individuos que más alimento consumieron. Además, la experiencia adquirida por el ratón de campo también influyó en la ingesta, reduciéndola en comparación con los individuos nuevos. Los niveles de testosterona fecal variaron la ingesta, al igual que las variables individuales sexo y estado reproductor. Así, los individuos con menores niveles de testosterona fecal, hembras y no reproductores, fueron los que más alimento consumieron. Finalmente, en relación con los factores ambientales, las variables humedad, temperatura y peso del individuo parecen predecir con una precisión del 98% la variación encontrada en los niveles de MCF del ratón de campo. Esto puede deberse al papel que juegan la humedad y la temperatura directamente en la cobertura vegetal e indirectamente en la disponibilidad de alimento y protección frente a los depredadores. Además, el peso parece estar relacionado con la condición corporal y con las relaciones intraespecíficas de dominancia y territorialidad. Por todo lo anterior, podemos concluir que el ratón de campo es capaz de

adaptar sus respuestas comportamental y fisiológica de estrés a los diferentes grados de riesgo de depredación percibidos, lo que se deriva de un mejor balance de los costes-beneficios asociados a cada situación que mejoran su eficacia biológica. Además, las variables ambientales humedad y temperatura y el factor individual peso del individuo parecen ser de destacada importancia a la hora de conocer la condición fisiológica de las poblaciones naturales. A este respecto, el ratón de campo constituye una especie clave en la cadena alimenticia de numerosos depredadores, lo que hace que conocer su estado fisiológico ante los diferentes cambios del medio sea de gran utilidad a la hora de proponer medidas de conservación y gestión para esta y otras poblaciones animales.

***Palabras clave*:** adaptación; factores ambientales; grados de riesgo de depredación; ingesta de alimento; metabolitos de corticosterona fecal; metabolitos de testosterona fecal; micromamíferos.

ABSTRACT

The environment in which animals live varies constantly. For this reason, animals such as small mammals have developed a wide range of morphological, behavioural and physiological adaptations to face the diversity of threats and risks that surround them. Thus, trigger the right response for each situation is a vital advantage to take into account, since it increases the probability of survival in the short (e.g. avoid the encounter with a predator) and long-term (e.g. avoid costly energy expenditure). Among the numerous dangers we have to stand out predation by directly affect prey species survival and environmental factors that influence the quality of the habitat and, therefore, in the resources it has to offer. Thereby, in this doctoral thesis we studied behavioural and physiological stress responses of wood mouse (*Apodemus sylvaticus* Linnaeus, 1758) to different degrees of predation risk by red fox (*Vulpes vulpes* Linnaeus, 1758) and to different environmental factors, analysing the influence of individual factors such as sex, reproductive status and acquired experience. In addition, we also investigated if wood mice modify food intake based on levels of faecal testosterone metabolites. Behavioural responses to predation risk, environmental factors and testosterone levels were studied by live trapping in two study areas, Monte de Valdelatas (Madrid, Spain) and Natural Park Os Montes do Invernadeiro (Galicia, Spain). For examining physiological responses, fresh faeces of animals captured were collected during the trapping sessions and the quantification of faecal corticosterone metabolites (FCM) and faecal testosterone metabolites (FTM) was carried out by an enzyme immunoassay. Results showed that wood mouse is able to estimate the degree of predation risk according to different

concentrations of faecal predator odour. In this regard, reproductive individuals were who most avoided entry into the traps with the maximum concentration of predator odour. Likewise, physiological stress response increased as increased predation risk perceived, being reproductive individuals who showed higher levels of FCMs. Food intake significantly increased as increased predator faecal odour too, reaching the highest value in the plot of maximum concentration. Females and non-breeding animals were individuals who most increased food intake. In addition, acquired experience also influenced food intake, reducing it in comparison with new individuals. Food intake varied to different levels of faecal testosterone metabolites and individual factors such as sex and reproductive status. Thus, animals with lower levels of faecal testosterone, females and non-breeding individuals, were those who consumed more food. Finally, in relation to environmental factors, humidity, temperature and body weight of wood mice seem to predict the variation of FCMs levels of wood mice with an accuracy of 98%. This may be due to the role played by humidity and temperature, both directly in the plant cover, and indirectly in the availability of food and protection from predators. In addition, body weight seems to be related to body condition and intraspecific relationships of dominance and territoriality. For all of the above, we can conclude that wood mice are capable of adapting their behavioural and physiological stress responses to different levels of predation risk perceived, which is derived from a better balance of the associated cost-benefit to each situation that enhance their biological effectiveness. In addition, environmental factors such as humidity and temperature and the individual factor body weight appear to be of great importance when it comes to know the

physiological condition of natural populations. In conclusion, wood mice is a very important prey for many predators, which makes that their physiological status is very useful when it comes to proposing measures of conservation and management for this and other animal populations.

Keywords: adaptation; degrees of predation risk; environmental factors; faecal corticosterone metabolites; faecal testosterone metabolites; food intake; small mammals.

COMUNICACIÓN QUÍMICA Y RIESGO DE DEPREDACIÓN

La eficacia biológica de cualquier ser vivo depende fundamentalmente de la capacidad que tenga para captar las señales comunicativas que se encuentran a su alrededor (Welton et al. 2003; Dall et al. 2005). La naturaleza de estas señales es muy variada y está adaptada al contexto en el que se desarrolla la comunicación, pudiendo agruparse en señales auditivas, táctiles, visuales y químicas (Eisenberg y Kleiman 1972). En un escenario nocturno y con abundante vegetación como es el medio en el que viven los mamíferos, las señales químicas se convierten en el modo más eficaz para la transmisión de la información (Ralls 1971; Eisenberg y Eisenberg 1981; Gorman y Trowbridge 1989). En los carnívoros, la señalización olorosa se define como la deposición de diferentes secreciones glandulares (Mykytowycz 1972; Albone y Perry 1976; Quay 1977; Asa et al. 1985), orina (Smith et al. 1989; Sillero-Zubiri y Macdonald 1998; Pal 2003) y heces en puntos estratégicos y objetos conspícuos (periferia del territorio, cruces de caminos y sustratos llamativos) que favorezcan la recepción del mensaje (Barrette y Messier 1980; Vilà et al. 1994; Monclús y de Miguel 2003; Barja et al. 2004; Barja 2005; Hebets y Papaj 2005; Barja 2009). La mayoría de estos compuestos químicos son utilizados por los carnívoros para el reconocimiento del entorno y la defensa del territorio y de las crías (Bowen y Cowan 1980; Gosling 1982; Gese y Ruff 1997; Hutchings y White 2000; Pal 2003; Barja y List 2006; Jordan et al. 2007; Piñeiro et al. 2012),

además de favorecer el reconocimiento individual al aportar información sobre el sexo, la edad, el estatus social y el estado reproductor (Stoddart y Sales 1985; Raymer et al. 1985; McEvoy et al. 2008; Barja et al. 2008; Barja et al. 2011).

Además de las numerosas funciones que posee el marcaje químico a nivel intraespecífico, éste tiene también un papel relevante en las relaciones interespecíficas (Gorman 1990). Así, los rastros olorosos depositados por los carnívoros suponen, al mismo tiempo, un sistema delator, informando de su presencia a sus potenciales presas, entre las que predominan los micromamíferos (Jones y Dayan 2000; Monclús et al. 2005; Fendt 2006; Navarro-Castilla y Barja 2014a).

La gran variedad de riesgos y peligros a los que tiene que hacer frente una presa a lo largo de su vida es muy amplia. Sin embargo, la depredación destaca por ser una de las principales fuerzas de selección (Lima y Dill 1990; Yin et al. 2011), ya que además de reducir numéricamente las densidades poblacionales de las especies presa (Brown et al. 1999; Hanski et al. 2001), desencadena cambios indirectos en el comportamiento, fisiología y morfología de las mismas, con unos costes tan necesarios como elevados (Lima y Dill 1990; Bouskila 1995; Dielenberg y McGregor 2001; Childress y Lung 2003; Apfelbach et al. 2005; Creel et al. 2007; Sherbrooke 2008; Rouco et al. 2011; Yin et al. 2011; Hegab et al. 2014a; Navarro-Castilla y Barja 2014a). Abrams (2000) define la depredación como el proceso en el que un depredador es capaz de matar y aprovechar energéticamente la materia de la especie presa. Para que esto sea posible deben darse cinco pasos previos por parte del depredador: detección, identificación, acercamiento, captura y consumo (Endler 1986; Sherbrooke 2008; Schmitz et al. 2013). Consecuentemente, las

presas han desarrollado un conjunto de defensas, no solo para evitar la detección inicial (cambios comportamentales o defensas morfológicas) (Lima 1998; Huffard 2006), sino mecanismos secundarios cuando la detección ya se ha realizado y la prioridad es evitar el consumo final (comportamiento de huida o sabores desagradables) (Huffard 2006; Luttbeg y Trussell 2013).

Dentro de las estrategias defensivas iniciales, la detección e identificación de las marcas olorosas de un depredador otorga a las presas una ventaja vital evitando, en última instancia, un encuentro que suele ser fatal para la presa (Edut y Eilam 2003). Esta capacidad ha sido observada en numerosas especies de micromamíferos (Calder y Gorman 1991; Barreto y Macdonald 1999; Apfelbach et al. 2005; Fendt 2006; Hegab et al. 2014c; Tortosa et al. 2015), incluida el ratón de campo (Dickman y Doncaster 1984; Navarro-Castilla y Barja 2014a; Navarro-Castilla y Barja 2014b), y parece desencadenarse como respuesta a la presencia de compuestos volátiles sulfurosos derivados de la digestión de proteínas animales (Nolte et al. 1994; Woolhouse y Morgan 1995). Sin embargo, existen numerosos factores, directos e indirectos, que pueden influir en la percepción por parte de las presas del riesgo de depredación al que están sometidas. Así, se ha observado que las presas no solo identifican las marcas fecales como un indicador directo de riesgo sino que son capaces de estimar y responder adecuadamente ante diferentes grados de riesgo de depredación a partir de la concentración de las señales químicas utilizadas por el depredador (Horat y Semlitsch 1994; Lima 1998; Dupuch et al. 2004; Kusch et al. 2004; Mirza et al. 2006; Hegab et al. 2014c). Esta idea, conocida como la hipótesis de evitación de las señales de amenaza de un depredador (*threat-sensitive predator*

avoidance hypothesis) (Helfman 1989), expone que las especies presa utilizan la concentración de las señales químicas del depredador para evaluar el peligro y responder acorde al riesgo percibido. Esta estrategia minimiza los costes asociados a evitar la depredación (ver respuestas comportamental y fisiológica) y permite a la presa ajustar su respuesta y aumentar así la probabilidad de supervivencia a corto y medio plazo. Del mismo modo, el riesgo de depredación percibido también varía dependiendo de factores indirectos como la existencia de refugios o la fase lunar, señales que en muchas ocasiones parecen aportar información más relevante que las señales directas (Orrock et al. 2004; Fletcher y Boonstra 2006b). En general, la escasa cobertura vegetal y la mayor visibilidad de las presas en las noches de luna llena aumentan el riesgo percibido, desencadenando la activación de la respuesta antidepredatoria (Kotler et al. 1991; Perea et al. 2011; Penteriani et al. 2013; Navarro-Castilla y Barja 2014b; Navarro-Castilla et al. 2017a).

RESPUESTA COMPORTAMENTAL ANTE DIFERENTES GRADOS DE RIESGO DE DEPREDACIÓN

Los cambios comportamentales son una de las estrategias antidepredatorias más comunes en los micromamíferos (Apfelbach et al. 2005). Estos cambios incluyen la evitación directa del olor del depredador (Dickman y Doncaster 1984; Bramley et al. 2000; Navarro-Castilla y Barja 2014b) y modificaciones en sus actividades diarias de forrajeo (Fenn y Macdonald 1995; Díaz et al. 2005; Verplancke et al. 2010), sociabilidad (Lipetz y Bekoff 1982; Alados 1985), reproducción (Creel et al. 2007) y uso del hábitat (Jędrzejewski y Jędrzejewska 1990; Jacob y Brown 2000; Creel et al. 2005) entre

otros (ver Lima y Dill 1990). Además, el contexto en el que se produzca el encuentro con el depredador va a influir en gran medida en la respuesta comportamental activada por la presa. Así, la presencia de un refugio cercano (Blanchard y Blanchard 1989), de una cobertura vegetal abundante (King 1985; Torre et al. 2002) o el tipo de estrategia que utilice el depredador (Jędrzejewski et al. 1993) modifica el tipo de comportamiento que deban mostrar.

El forrajeo es una actividad energéticamente necesaria pero que expone a la presa ante los depredadores. Ajustar el comportamiento de forrajeo espacial (en las zonas más seguras) y temporalmente (cuando el depredador no está presente) (Fenn y Macdonald 1995; Díaz et al. 2005), reduce la posibilidad de ser depredado mientras satisfaces el resto de necesidades vitales. Además, otros factores ambientales como la existencia de una densa cobertura vegetal, una abundante nubosidad o una escasa iluminación lunar mantienen los niveles de forrajeo adecuados al reducirse el riesgo de depredación percibido (Orrock et al. 2004; Kotler et al. 2010; Wróbel y Bogdziewicz 2015; Busch y Burroni 2015).

El coste de estas estrategias defensivas no solo implica una reducción de los ingresos energéticos al reducir el tiempo de forrajeo, sino que también supone a las presas una inversión energética en la formación de estructuras defensivas, un menor éxito en el apareamiento, una mayor vulnerabilidad a otros depredadores e incluso la emigración a otros territorios más seguros (Preisser et al. 2005). De acuerdo con la hipótesis de asignación de riesgos (*risk allocation hypothesis*) propuesta por Lima y Bednekoff (1999), los animales destinan más tiempo a alimentarse cuando el riesgo de depredación es bajo, reduciendo dicho tiempo cuando el riesgo

aumenta. Sin embargo, si el riesgo de depredación elevado se mantiene durante largos periodos de tiempo, la presa debe reanudar la alimentación manteniéndola en unos mínimos para la supervivencia, aumentándola en gran medida durante los cortos periodos de riesgo bajo. En conclusión, teniendo en cuenta los costes que implica la proyección de los comportamientos defensivos, las presas han desarrollado diferentes estrategias para estimar el grado de riesgo de depredación real y hacer balance de los costes y beneficios que supone en cada caso concreto la activación de la respuesta antidepredatoria (Ydenberg y Dill 1986; Kavaliers y Choleris 2001).

RESPUESTA FISIOLÓGICA DE ESTRÉS: DIFERENTES GRADOS DE RIESGO DE DEPREDACIÓN Y FACTORES AMBIENTALES

A pesar de que la falta de respuesta comportamental observada en ciertos estudios con micromamíferos (Busch y Burroni 2015) había sido juzgada como falta de reconocimiento ante el depredador, hoy en día los numerosos trabajos en los que interviene la fisiología animal sacan a la luz las respuestas fisiológicas que antes pasaban desapercibidas (Monclús et al. 2005). Este nuevo enfoque endocrino permite no solo evaluar las consecuencias a nivel evolutivo de la depredación sobre las poblaciones (Zanette et al. 2011), sino también analizar el efecto que tienen las alteraciones en el medio natural (sequías, deforestación, fragmentación del hábitat y cambio climático, entre otros) sobre la conservación de las especies (Mazerolle y Hobson 2002; Suorsa et al. 2004; Brown y Fuller 2006).

Selye (1960) define estrés como el conjunto de cambios fisiológicos y neurológicos que se producen en un organismo como respuesta ante un estímulo nocivo. El hábitat de los seres vivos no es estático y por ello los seres vivos tienen que interpretar y adaptarse a los diferentes estímulos que reciben, considerándose nocivos aquellos que alteren la homeostasis del organismo (Sapolsky 2002; Wingfield 2005; Barja 2015). Estos estímulos pueden ser internos (traumatismos, dolor, miedo, hambre, ansiedad) o externos (estatus social, temperaturas extremas, presencia de un depredador, perturbaciones en el medio, contaminación) y reciben el nombre de agentes estresantes o estresores (Romero 2004). Así, cuando un ser vivo se enfrenta a un agente estresante se activa un conjunto de reacciones endocrinas conocidas como respuesta fisiológica de estrés, con el objetivo final de recuperar la homeostasis. La respuesta fisiológica de estrés conlleva la activación del eje corticotropo (HPA: Hipotálamo-Pituitaria-Adrenal) (Axelrod y Reisine 1984; Stewart 2003). El hipotálamo recibe el agente estresante y, como respuesta, segrega el factor liberador de corticotropina (CRF), que estimula a su vez la liberación de la hormona adrenocorticotropina (ACTH) en la pituitaria. La ACTH viaja por el torrente sanguíneo hasta la corteza de las glándulas suprarrenales donde activará la producción y liberación de hormonas esteroideas, entre las que se encuentran los glucocorticoides (GCs: cortisol / corticosterona en roedores) (Sapolsky et al. 2000; Melmed y Kleinberg 2003; Barja 2015) (Fig. 1). El hígado y/o riñón son los órganos encargados de metabolizar las hormonas esteroideas liberadas a la sangre y que posteriormente serán excretadas en la orina o la bilis. De la bilis, los metabolitos de dichas hormonas pasarán al tracto digestivo, para ser eliminadas finalmente

por las heces (Bamberg et al. 2001; Möstl y Palme 2002; Barja 2015). La función principal de los GCs es movilizar las reservas de glucosa y aumentar su concentración en sangre para aportar la energía necesaria a los órganos encargados de hacer frente al agente estresante (Raynaert et al. 1976; Sapolsky 1994a). Cuando el agente estresante cesa o su acción ha sido controlada por el organismo, la liberación de CGs se interrumpe por un proceso de inhibición negativa (Fig. 1) al suspenderse la liberación de CRF y ACTH (Randall et al. 1998; Sapolsky et al. 2000).

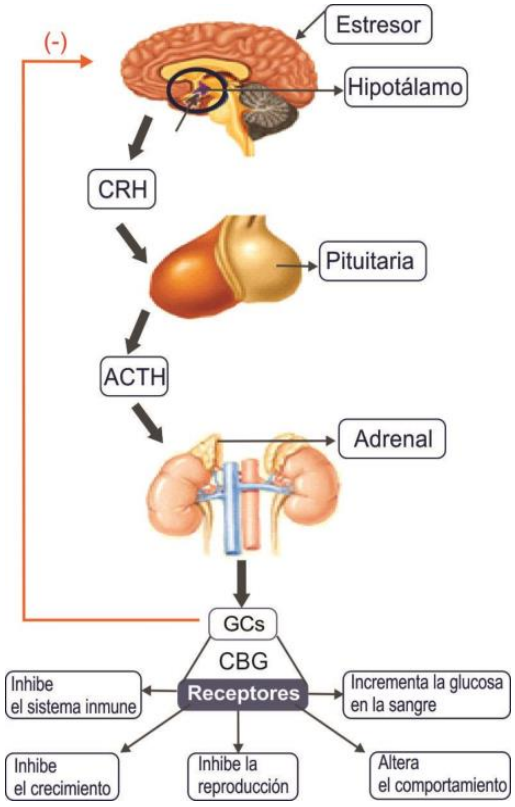


Figura 1. Esquema de la respuesta fisiológica de estrés en mamíferos (tomado de Romero 2004).

La respuesta fisiológica de estrés es un proceso secuencial con diferentes efectos dependiendo de la duración que tenga el agente estresante. Según Selye (1936) los cambios endocrinos van variando a lo largo del proceso fisiológico denominado *Síndrome de Adaptación General* (SAG), el cual se desarrolla en 3 fases con diferentes repercusiones para el organismo. La primera fase se denomina *Reacción de emergencia o reacción adrenal* y se caracteriza por ser una respuesta de efecto inmediato ante la presencia de un estresor. Así, el cuerpo se prepara para el gasto energético que conlleva hacer frente a la situación nociva (activación del sistema simpático, liberación de adrenalina y aumento de la frecuencia respiratoria y cardiaca). Si con esta respuesta el organismo no ha conseguido frenar la acción del estresor, se activa la segunda fase denominada *Estado de resistencia*. En esta fase se produce la activación del eje corticotropo HPA que desencadena la liberación de la ACTH y, por consiguiente, el aumento en los niveles de glucocorticoides en sangre. Hasta este punto, la activación del eje HPA es una respuesta adaptativa y por lo tanto beneficiosa, ya que aumentan las posibilidades que tiene el organismo de sobrevivir (Wingfield y Romero 2001) al aportar rápidamente energía extra para acciones como huir de un depredador (Boonstra et al. 1998; Monclús et al. 2008) o mejorar la eficacia en el apareamiento (Bauer et al. 2008). Sin embargo, si el estresor se alarga en el tiempo, el estrés se considera crónico y el organismo entra en la tercera fase denominada de *Estado de agotamiento.* En esta última fase, los elevados niveles de glucocorticoides alcanzados pueden desencadenar importantes patologías en el individuo como reducción de la capacidad reproductora/infertilidad, supresión del sistema inmune, falta de resistencia a parásitos, úlceras e inhibición del

crecimiento, entre otras (Munck et al. 1984; Sapolsky 1994b; Sapolsky et al. 2000; Stewart 2003; Romero 2004; Bortolotti et al. 2009; Henry y Stephens 2013). La mayoría de estas patologías comprometen el mantenimiento a largo plazo de las poblaciones ya que afectan directamente a la eficacia biológica de los organismos (Wikelski et al. 2001; Möstl y Palme 2002).

Actualmente, numerosos estudios utilizan los niveles de GCs como indicadores del estado fisiológico de estrés de un organismo (Harper y Austad 2000; Wingfield y Romero 2001; Wikelski et al. 2001; Scheuerlein et al. 2001; Möstl y Palme 2002; Mateo 2006; Barja et al. 2007). El nivel de GCs se puede medir en plasma (Wikelski et al. 2001; Bauer et al. 2008), orina y heces (Teskey-Gerstl et al. 2000; Bosson et al. 2009). A pesar de su amplio uso en el pasado (Broom y Johnson 1993), los niveles de GCs en plasma están influenciados por las condiciones de muestreo, ya que la obtención de este tipo de muestras requiere la captura, punción y manejo del animal (De Villiers et al. 1997; von der Ohe et al. 2004; Young et al. 2004), lo que se ha demostrado que induce en sí mismo la activación de las respuestas fisiológicas (Kenagy y Place 2000; Place y Kenagy 2000; Cook et al. 2000). Además, los niveles de GCs en plasma presentan variaciones debidas a la secreción pulsátil de las hormonas y a la influencia del ciclo circadiano (Van Cauter et al. 1996). En contraposición a esta técnica, el análisis de los metabolitos de glucocorticoides fecales (MGFs) es un método no invasivo, que minimiza las perturbaciones asociadas a la obtención de muestras producidas por otros métodos (Barja et al. 2012). En los últimos años, este método ha sido ampliamente utilizado para analizar el estado fisiológico de estrés animal en una gran variedad de especies de

mamíferos (Dehnhard et al. 2001; Young et al. 2004; Millspaugh y Washburn 2004; von der Ohe et al. 2004; Ylönen et al. 2006; Charbonnel et al. 2007; Barja et al. 2012; Piñeiro et al. 2012).

Entre las causas por las que un animal activa la respuesta fisiológica de estrés se encuentran factores humanos como la presión turística (Barja et al. 2007; Piñeiro et al. 2012), la práctica de deportes al aire libre (Arlettaz et al. 2007; Thiel et al. 2008), la caza (Bateson y Bradshaw 1997; Tarjuelo et al. 2015; Casas et al. 2016) o el tráfico (Millspaugh et al. 2001; Rolland et al. 2012; Navarro-Castilla et al. 2014a), y factores ambientales entre los que se incluyen la depredación y factores climáticos. Así, se ha observado como la presencia de un depredador induce la liberación de GCs en numerosas especies presa (Boonstra et al. 1998; Eilam et al. 1999; Monclús et al. 2008; Preisser 2009; Sheriff et al. 2011a), pudiendo adaptar esta respuesta en función de la historia evolutiva presa-depredador (Hegab et al. 2014a) y del grado de riesgo percibido a través de las señales químicas depositadas por el depredador (Hegab et al. 2014c).

Las variables ambientales como temperatura, pluviosidad, altitud, espesor de la cobertura vegetal y disponibilidad de alimento también son factores de gran importancia a la hora de analizar el bienestar animal (Escribano et al. 2009) ya que determinan la calidad del hábitat en el que se va a desarrollar la especie. En los mamíferos en general, la falta de precipitaciones, vinculada directamente con la escasez de alimento disponible (Díaz et al. 2010), está relacionada con un aumento de los niveles de GCs (Sheriff et al. 2012), al igual que las temperaturas extremas (Millspaugh et al. 2001; Sheriff et al. 2012). La altitud también está correlacionada con el aumento de la respuesta fisiológica de estrés en mamíferos (Sheriff et al. 2012). Estos

resultados coinciden con la preferencia observada en numerosas especies de micromamíferos por hábitats de buena cobertura vegetal debido a que dichos hábitats les ofrecen protección frente a los depredadores, refugio ante las condiciones climáticas adversas y una amplia variedad de recursos tróficos (Tew y Macdonald 1993; Ouin et al. 2000; Orrock et al. 2004).

En conclusión, el ambiente en el que viven los mamíferos está sujeto a numerosos cambios, algunos predecibles pero muchos impredecibles, que pueden alterar significativamente la biología de la especie. Los organismos están preparados para responder comportamental y/o fisiológicamente ante dichos cambios, adaptándose a las nuevas condiciones. Sin embargo, el elevado gasto energético que a veces supone hacer frente a un estresor (Creel 2001), y su permanencia en el tiempo, puede llegar a comprometer la supervivencia de la especie a largo plazo (Möstl y Palme 2002). Para evitar que esto suceda surge la Fisiología de la Conservación, una disciplina dentro de la Biología que se encarga de conocer cómo responde cada grupo animal ante los diferentes estresores, lo que es fundamental para tomar las medidas adecuadas antes de que se produzca un daño irreversible y asegurar así la conservación de las especies.

ESPECIE MODELO: EL RATÓN DE CAMPO (*Apodemus sylvaticus*)

El género *Apodemus* está integrado por 15 especies (Nowak 1999; Liu et al. 2004) de las cuales solo dos se encuentran en la Península Ibérica, el ratón de campo y el ratón leonado (*Apodemus flavicollis* Melchior, 1834), dos especies del subgénero *Sylvaemus* (Michaux et

al. 2002; Filippucci et al. 2002; Hoofer et al. 2007) con área de simpatría en el tercio norte peninsular (Arrizabalaga y Torre 2007). El ratón de campo es un roedor de tamaño pequeño (peso corporal máximo 40 g) (Blanco 1998) fácilmente distinguible del resto de Múridos por su cabeza alargada de gran tamaño en la que destacan unos ojos negros oscuros y orejas redondeadas. Además, presenta las extremidades posteriores notablemente más largas que las delanteras, lo que le permite incorporar saltos en sus movimientos, y una cola delgada de longitud igual o superior a la longitud cabeza-cuerpo, con una cubierta tegumentaria que se libera con facilidad (Jubete 2002; Torre et al. 2002). La coloración del pelaje varía con la edad, siendo dorsalmente grisácea en los juveniles hasta el primer o segundo mes de vida en el que experimentan su primera muda, momento a partir del cual se va tornando a colores rojizos. El color del vientre pasará de blanco a amarillento con el paso del tiempo (Sans-Coma et al. 1987; Torre et al. 2002). Los machos son ligeramente de mayor tamaño que las hembras, aunque el dimorfismo sexual es leve (Alcántara y Díaz 1996).

Es una especie de hábitos nocturnos (Montgomery y Gurnell 1985), aunque en ocasiones puede estar activo también durante el día. Su patrón de actividad se caracteriza por ser bifásico en verano presentando dos picos de máxima actividad entre las dos y cuatro horas siguientes al anochecer y entre las tres y cinco horas previas al amanecer. En invierno el patrón es monofásico debido al acortamiento de las noches, alcanzando su máximo de actividad en las horas medias de la noche (Wolton 1983). El periodo reproductor varía entre las zonas del norte y sur de la Península. En las zonas septentrionales, los ratones de campo están activos sexualmente entre los meses de

primavera y otoño, sufriendo una latencia en los meses fríos de invierno (Clarke 1985). Por el contrario, la máxima actividad reproductora en las zonas meridionales se concentra en los últimos meses de invierno y la totalidad de la primavera, con el periodo de latencia localizado en verano (Fons y Saint Girons 1993). La gestación tiene una duración entre 20 y 32 días (Clarke 1985) y el número medio de crías por parto es 5,5 (Sans-Coma et al. 1987).

Según Guisan y Zimmermann (2000) la distribución de las especies está determinada por los requerimientos específicos de la especie y por los factores ambientales directos e indirectos del área en la que habite. Las características del ratón de campo lo convierten en una especie de amplia distribución, propia de la región Paleártica occidental. Las citas lo sitúan distribuido homogéneamente por toda Europa hasta la mitad sur de Noruega y Suecia, Asia Menor y norte de África, además de en la mayoría de islas como Islandia, Islas Británicas y casi todas las islas del Mediterráneo, a excepción de las islas atlánticas de Canarias y Azores (Montgomery 1999; Torre et al. 2002; Wilson y Reeder 2005; Díaz et al. 2010) (Fig. 2).

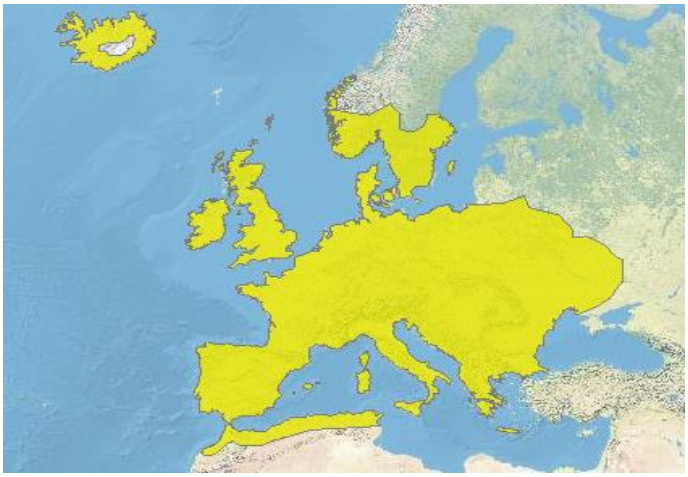


Figura 2. Área de distribución del ratón de campo en Europa (tomada de Schlitter et al. 2008)**.**

En España se trata de la especie de micromamífero más abundante encontrándose en la totalidad del territorio peninsular, así como en las islas Baleares, Ceuta y Melilla (Alcántara y Tellería 1991; Torre et al. 2002) (Fig. 3).

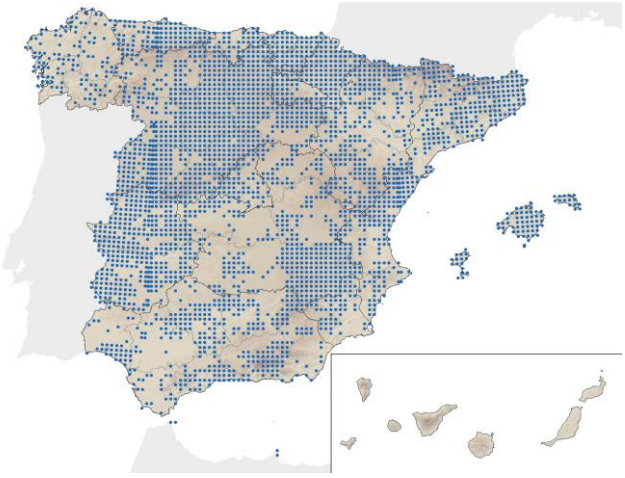


Figura 3. Distribución del ratón de campo en España (tomada de Palomo y Gisbert 2002).

Se trata de una especie generalista, capaz de adaptarse a una gran variedad de hábitats desde el nivel del mar hasta los 2500 m de altitud (Wolton y Flowerdew 1985; Zhang y Usher 1991; Gorman et al. 1993; Tew y Macdonald 1994; Montgomery 1999). Sin embargo, numerosos estudios muestran una clara selección positiva por las zonas con buena cobertura vegetal (Tew y Macdonald 1993; Montgomery y Dowie 1993; Todd et al. 2000; Ouin et al. 2000; Orrock et al. 2004; Navarro-Castilla y Barja 2014a; Navarro-Castilla et al. 2017b) como los bosques de hoja perenne y matorrales de la región Mediterránea del sur de Europa (Torre et al. 2002).

El área de campeo del ratón de campo varía dependiendo de factores como la estacionalidad (Todd et al. 2000), el tipo de hábitat (Attuquayefio et al. 1986; Akbar y Gorman 1993), el estado reproductor y el sexo (Attuquayefio et al. 1986; Tew y Macdonald 1994; Corp et al. 1997). En cuanto al sexo, los machos poseen áreas de campeo mayores que las hembras debido a su comportamiento territorial, además de aumentar dichas áreas durante la época reproductora para ambos sexos. En particular, la superficie del área de campeo en ambientes forestales oscila entre los 3500 m^2 durante la época reproductora y los 1000 m^2 en época no reproductora (Corp et al. 1997). La calidad del hábitat también es un factor determinante ya que la ausencia de recursos disponibles en los hábitats de baja calidad obliga a los individuos a recorrer distancias mayores para conseguir una cantidad de alimento suficiente para satisfacer sus requerimientos energéticos (Akbar y Gorman 1993). En comparación con el ambiente forestal antes expuesto, el área de campeo del ratón de campo puede aumentar hasta 55.000 m^2 en ambientes dunares, medios caracterizados por la baja tasa de producción (Corp et al. 1997).

Su alta capacidad de adaptación también se ve reflejada en su dieta omnívora, consumiendo una gran variedad de semillas y frutos, generalmente hayucos y bellotas, e incluso algún tipo de presa viva como insectos (Górecki y Gębczyńska 1962; Watts 1968; Obrtel y Holisova 1979; Torre et al. 2002).

Diversos estudios de ecología trófica sitúan al ratón de campo como uno de los micromamíferos más consumidos por un amplio rango de depredadores. Destaca la presencia de este roedor en la dieta de especies como la gineta (*Genetta genetta* Linnaeus, 1758) (Virgós et al. 1999; Rosalino y Santos-Reis 2002; Torre et al. 2003), el zorro rojo (Blanco 1998; Goldyn et al. 2003), la marta europea (*Martes martes* Linnaeus, 1758) (Clevenger 1993; Rosellini et al. 2008) y el gato montés (*Felis silvestris* Schreber, 1777) (Piñeiro y Barja 2011). Estos depredadores, como la mayoría de los carnívoros, son animales de hábitos nocturnos que cazan principalmente al atardecer y al amanecer, por lo que coinciden en el tiempo con sus presas (Doncaster y Macdonald 1997; Torre et al. 2002; Camps 2008). Además, también es importante la tasa de depredación por rapaces nocturnas y diurnas con actividad crepuscular y matutina (Halle 1988), ofidios y ocasionalmente otras especies no especializadas como el jabalí (*Sus scrofa* Linnaeus, 1758), la garza real (*Ardea cinearea* Linnaeus, 1758) y la cigüeña blanca (*Ciconia ciconia* Linnaeus, 1758).

MONTE DE VALDELATAS (MADRID)

El trabajo de campo relativo a los capítulos I, II y III de la presente Tesis Doctoral ha sido realizado en el Monte de Valdelatas (Madrid, España). El Monte de Valdelatas (Fig. 4) ocupa una extensión de 290 ha divididas entre los municipios de Alcobendas (90 ha) y Madrid (200 ha) (Comunidad Autónoma de Madrid; 40°32'4"N - 3°40'46"W). Está catalogado como Monte Preservado según la ley 16/1995 de la Comunidad de Madrid y se considera una prolongación natural del Monte de El Pardo, del que se separa por el paso de la M-607 y las vías ferroviarias. Presenta un relieve poco acusado, con una altitud media de 650 m.s.n.m., y con un curso permanente de agua no potable procedente del Arroyo de la Almenara.

Figura 4. Localización y vista aérea del área de estudio (línea amarilla) en el Monte de Valdelatas (Madrid).

El Monte de Valdelatas presenta un clima mediterráneo continental, caracterizado por una marcada sequía estival y unos inviernos secos y moderadamente fríos. La temperatura media anual oscila entre 14,5ºC y 15ºC, situándose las mínimas en los meses invernales de diciembre y enero, y las máximas durante el verano (julio y agosto). La amplitud térmica diaria en la zona de la periferia donde se encuentra el área de estudio llega a ser de más de 13ºC. Las precipitaciones son escasas, con una media anual de 400 mm, siendo la gran parte de ella en forma de lluvia y en los meses de otoño. Presenta un 57 % de humedad media anual, lo que la convierte en la capital de provincia más seca de España en cuanto a humedad relativa se refiere.

La comunidad vegetal predominante en todo el área de estudio es el bosque mediterráneo que está constituido principalmente por encinares densos (*Quercus ilex*) y pinares de repoblación de pino marítimo (*Pinus pinaster*) y pino piñonero (*Pinus pinea*) (Fig. 5). El matorral está formado fundamentalmente por asociaciones de jara pringosa (*Cistus ladanifer*), jarilla (*Halimium umbellatum*), tomillo (*Thymus zygis*), y zancajareña (*Artemisia campestris*). En las orillas de los arroyos, encontraremos vegetación de ribera como el aliso común (*Alnus glutinosa*), álamo blanco (*Populus alba*) y sauce blanco (*Salix alba*).

Figura 5. (A) Vista general de la vegetación del Monte de Valdelatas formada principalmente por encinas y pinos de repoblación. Autor: Á. Navarro-Castilla; **(B)** Detalle de la vegetación del área donde se llevó a cabo el trabajo experimental caracterizada por amplias extensiones de encinar con sotobosque de herbáceas y jaras. Autora: B. Sánchez-González.

La comunicación existente con el Monte El Pardo favorece la presencia de una rica y variada lista de especies animales en el Monte de Valdelatas. Entre las aves más significativas se encuentran palomas torcaces (*Columba palumbus* Linnaeus, 1758), urracas (*Pica pica* Linnaeus, 1758) y aves insectívoras como el carbonero común (*Parus major* Linnaeus, 1758), el herrerillo (*Cyanistes caeruleus* Linnaeus, 1758), el pito real (*Picus viridis* Linnaeus, 1758), la oropéndola (*Oriolus oriolus* Linnaeus, 1758) y la abubilla (*Upupa epops* Linnaeus, 1758), entre otros (Villarán et al. 2005). Además, también están presentes algunas rapaces como la lechuza común (*Tyto alba* Scopoli, 1769), el mochuelo europeo (*Athene noctua* Scopoli, 1769) y

el milano real (*Milvus milvus* Linnaeus, 1758). Entre los reptiles destaca la presencia del lagarto ocelado (*Timon lepidus* Daudin, 1802), la culebra de escalera (*Rhinechis scalaris* Schinz, 1822) y la culebra bastarda (*Malpolon monspessulanus* Hermann, 1809).

El área de estudio también cuenta con una rica comunidad de mamíferos como demuestran los datos obtenidos en estudios previos en el mismo área (Monclús y de Miguel 2003; Monclús et al. 2009; Navarro-Castilla y Barja 2014a). Es significativa la presencia de carnívoros como el zorro rojo (Fig. 6A) y la gineta (Fig. 6B) y ungulados como el corzo (*Capreolus capreolus* Linnaeus, 1758) y el jabalí. Los micromamíferos también son muy abundantes, destacando la presencia de la ardilla roja (*Sciurus vulgaris* Linnaeus, 1758), el conejo europeo (*Oryctolagus cunniculus* Linnaeus, 1758) (Fig. 7A), el ratón de campo (Fig. 7B), el ratón moruno (*Mus spretus* Linnaeus, 1758) (Fig. 7C) y el topillo campesino (*Microtus arvalis* Linnaeus, 1758) (Fig. 7D).

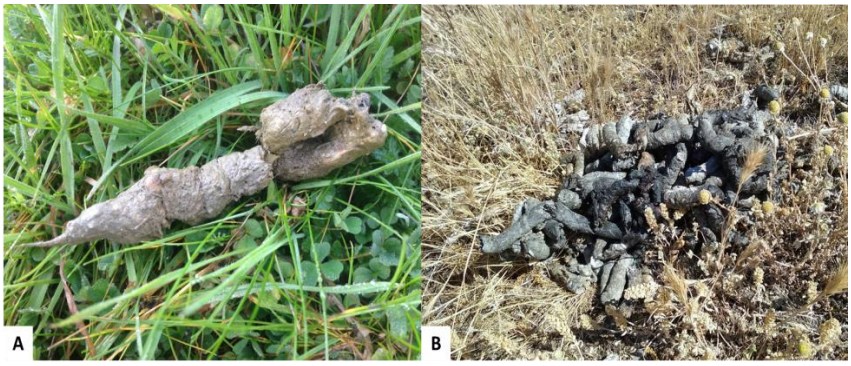


Figura 6. Excrementos de zorro **(A)** y letrina de gineta **(B)** detectadas en el área de estudio. Autores: B. Sánchez-González y F.J. de Miguel.

Figura 7. Letrina de conejo **(A)**, ratón de campo **(B)**, ratón moruno **(C)** y topillo campesino **(D)** fotografiados en el área de estudio durante los trampeos de vivo. Autores: B. Sánchez-González y Á. Navarro-Castilla.

PARQUE NATURAL OS MONTES DO INVERNADEIRO (OURENSE)

El área de estudio donde se llevaron cabo los experimentos relativos al capítulo IV es el Parque Natural Os Montes do Invernadeiro (Ourense, España). O Invernadeiro está catalogado como Parque Natural desde el 5 de junio de 1997 y pertenece al término municipal de Vilariño de Conso (42º 07' 59.6" N - 7º 19' 28.2" W). Este espacio montañoso está situado al sur del Macizo Central Orensano y está formado por las sierras de San Mamede, Queixa, Manzaneda y el Fial das Corzas (Fig.

8). Ocupa una extensión de 5.722 ha con elevaciones entre los 880 m y los 1.782 m de altitud alcanzados en la estación de montaña de Manzaneda, en la Sierra de Queixa. El Parque cuenta con aporte de agua permanente gracias al paso de los ríos Ribeira Grande al norte y Ribeira Pequeña al sur, pertenecientes a la cuenca del Sil, encargados del modelado en forma de V pronunciada de sus respectivos valles. Esta orografía tan abrupta es consecuencia del modelado glaciar ocurrido en el pasado, gracias al cual hoy en día se pueden observar un circo glaciar y numerosas cascadas.

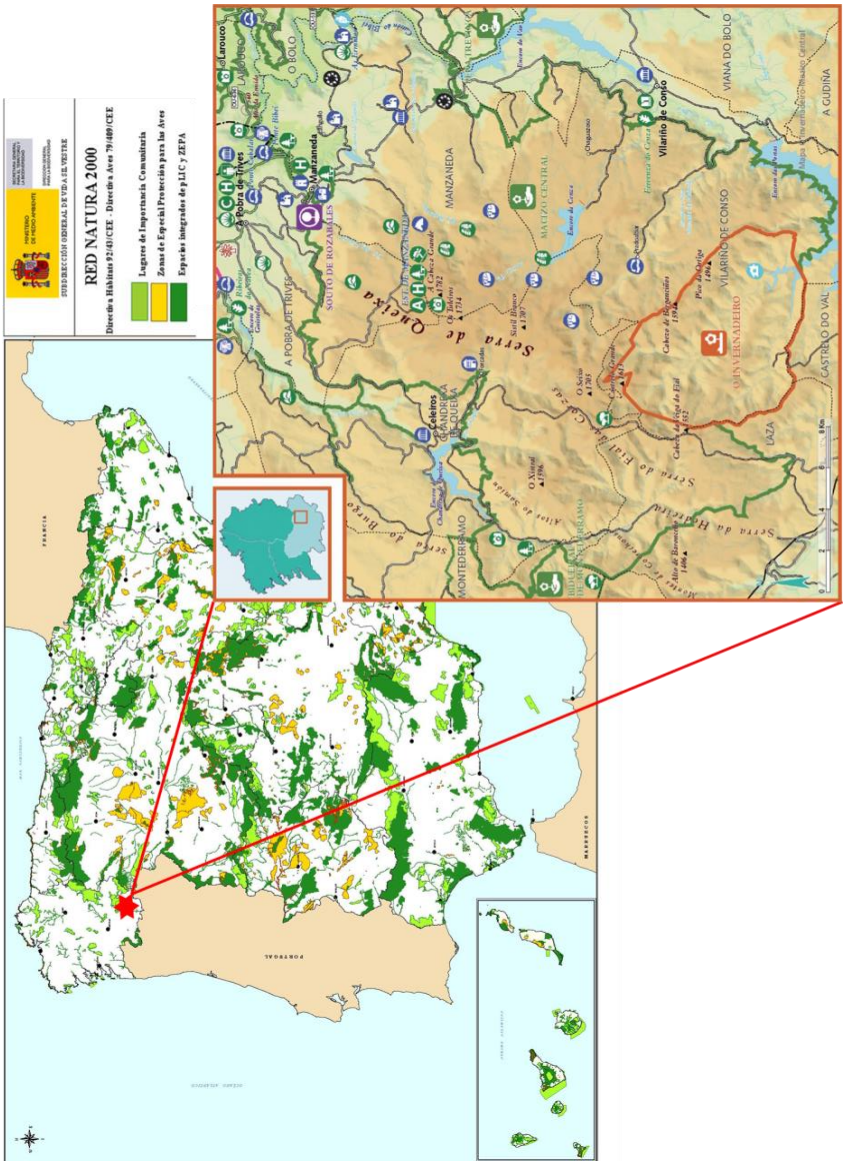


Figura 8. Situación geográfica del Parque Natural Os Montes do Invernadeiro en la Península Ibérica.

Debido a su localización de interior, este Parque no participa en su totalidad de las condiciones del clima oceánico, presentando

unas características más similares a las del clima continental con influencias atlánticas y mediterráneas. Así, las temperaturas experimentan importantes variaciones a lo largo del año siendo suaves en verano con medias de 15ºC y frías en invierno pudiendo bajar hasta los -7ºC. La precipitación media anual es de 2000 mm, fundamentalmente en forma de abundantes lluvias y nieve.

La vegetación predominante del territorio es el matorral mixto formado por brezo rubio (*Erica australis*), carquesia (*Genista tridentata*), jaguarzo (*Halimium lasianthum*) y, en las zonas más húmedas, retama blanca (*Genista florida*) y escoba blanca (*Cytisus multiflorus*). El bosque atlántico autóctono se encuentra fundamentalmente conservado en los valles y riberas y está formado por asociaciones de roble común (*Quercus robur*), abedul (*Betula celtibérica*) y acebo (*Ilex aquifolium*). También existen formaciones de roble melojo (*Q. pirenaica*) y, de forma más esporádica, de tejo común (*Taxus baccata*), prueba de la influencia del clima mediterráneo. A medida que se asciende en altitud el paisaje cambia y predominan las praderas con árandanos (*Vaccinium myrtillus*) y quiruela (*Erica umbellata*) (Castroviejo 1977). Además, existen grandes extensiones ocupadas por repoblaciones de pino albar (*Pinus sylvestris*) y pino marítimo (*P. pinaster*) (Barja 2001; Pulgar 2004) (Fig. 9).

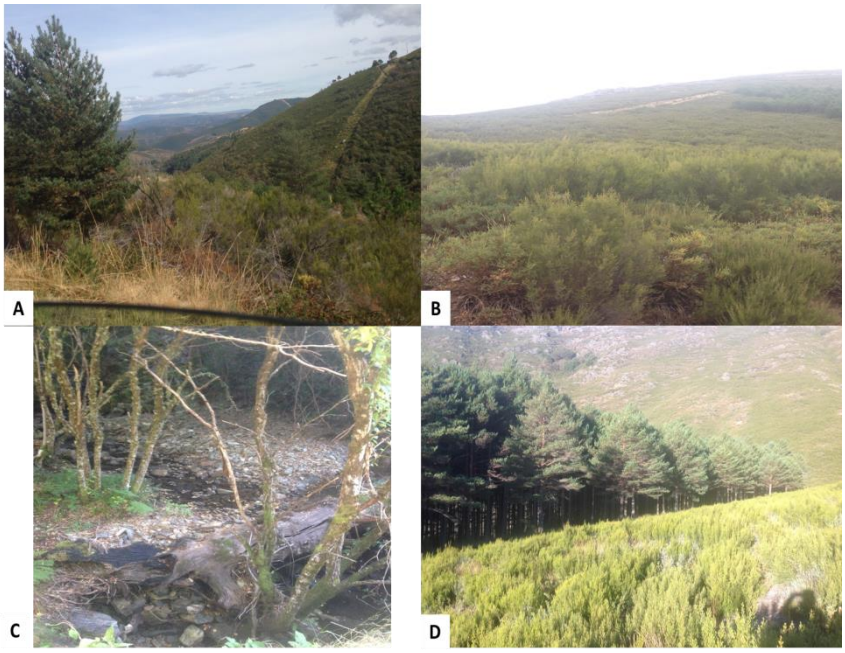


Figura 9. Hábitats predominantes en el Parque Natural Os Montes do Invernadeiro. **(A)** Vista general del área de estudio; **(B)** Matorral; **(C)** Bosque caducifolio asociado a valles y vaguadas; **(D)** Repoblaciones de pino albar y pino marítimo. Autora: B. Sánchez-González.

El Parque Natural cuenta con una rica diversidad faunística en la que destaca, en lo alto de la cadena trófica, la presencia de una población asentada de lobo ibérico (*Canis lupus* Linnaeus, 1758) (Fig. 10A) (Barja y Rosellini 2008; Barja et al. 2008), además de numerosos mesocarnívoros como la nutria (*Lutra lutra* Linnaeus, 1758), la marta (Fig. 10B), la comadreja (*Mustela nivalis* Linnaeus, 1766), el tejón (*Meles meles* Linnaeus, 1758), la gineta, el gato montés (*Felis silvestris* Schreber, 1777), el zorro, el turón (*Mustela putorius* Linnaeus, 1758) y el armiño (*Mustela erminea* Linnaeus, 1758) (Rosellini et al. 2008; Piñeiro et al. 2012).

Figura 10. Excrementos de lobo ibérico **(A)** y ejemplar de marta europea fotografiada mediante fototrampeo **(B)** presentes en el área de estudio. Autoras: B. Sánchez-González y M.C. Hernández.

Entre los micromamíferos destaca la presencia del ratón de campo (Fig. 11A) como una de las especies más abundantes en todos los hábitats, seguida de varias especies del género *Mus*, el lirón gris (*Glis glis* Linnaeus, 1766), la ardilla roja, el lirón careto (*Eliomys quercinus* Linnaeus, 1766) (Fig. 11B), el erizo común (*Erinaceus europaeus* Linnaeus, 1758) (Fig. 11C), la musaraña enana (*Sorex minutus* Linnaeus, 1766), la musaraña común (Fig. 11D) (*Crocidura russula* Hermann, 1780) y varias especies de topillos como el topillo nival (*Chionomys nivalis* Martins, 1842), el topillo agreste (*Microtus agrestis* Linnaeus, 1761) y el topillo de cabrera (*Microtus cabrerae* Thomas, 1906) (Rosellini et al. 2008; Piñeiro y Barja 2011).

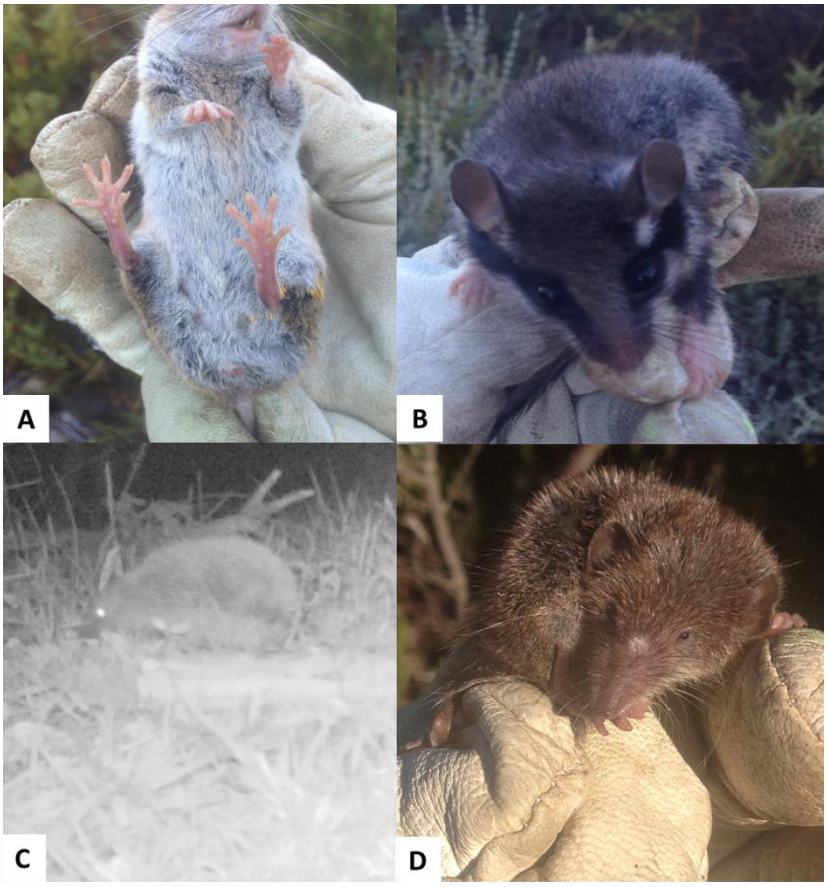


Figura 11. Ratón de campo (A), lirón careto (B), erizo común (C) y musaraña común (D) fotografiados en el área de estudio. Autoras: B. Sánchez-González y M.C. Hernández.

Igual de importante es la presencia del águila real (*Aquila chrysaetos* Linnaeus, 1758), el halcón peregrino (*Falco peregrinus* Tunstall, 1771), el águila culebrera (*Circaetus gallicus* Gmelin, 1788) y el aguilucho cenizo (*Circus pygargus* Linnaeus, 1758) como rapaces características, así como de ungulados como el corzo (*Capreolus capreolus* Linnaeus, 1758), el ciervo (*Cervus elaphus* Linnaeus, 1758) y el jabalí.

Debido a su elevado valor faunístico, botánico, geomorfológico y paisajístico, y con el objetivo de asegurar su conservación, el Parque Natural Os Montes do Invernadeiro se divide en tres zonas con diferentes restricciones de uso (Fig. 12). (1) *Zona de uso público restringido* (2.466 ha): está permitido el paso de visitantes tanto a pie como en coche. En esta zona se encuentran las aulas de la naturaleza donde se hacen numerosas visitas de escolares e investigadores, siendo posible pernoctar en ellas con el permiso oportuno (2) *Zona de protección especial* (2.142 ha): restringida a visitantes que hagan la ruta a pie y siempre guiados por personal de la Xunta de Galicia. (3) *Zona de reserva integral* (1.114 ha): zona exclusiva para investigadores y personal del parque. Independientemente de la zona que vayas a visitar, todos los visitantes deberán solicitar el permiso de entrada en la Delegación Provincial de la Consellería de Medio Ambiente e Ordenación do Territorio de Ourense, con un aforo máximo de 50 personas por día.

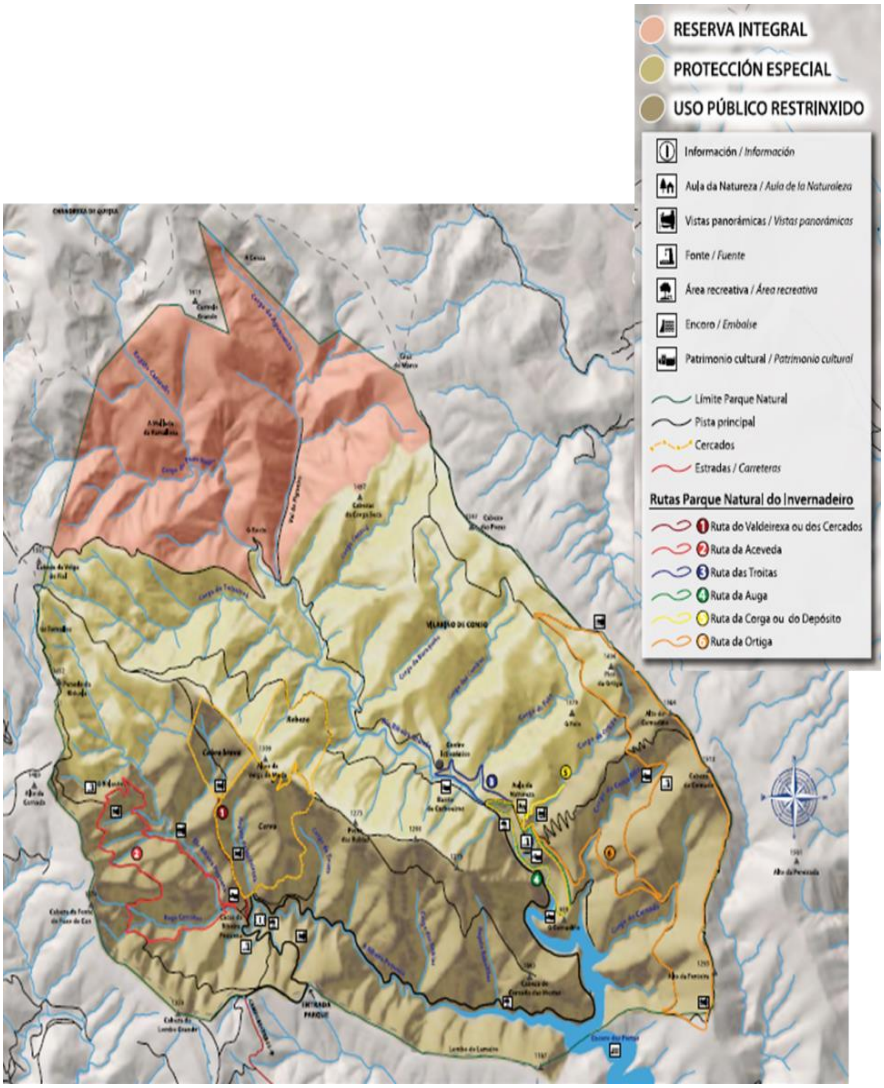


Figura 12. Mapa del Parque Natural Os Montes do Invernadeiro dividido en las tres zonas de protección (tomado de la página web de la Xunta de Galicia http://parquesnaturais.xunta.gal/es/o-invernadeiro).

OBJETIVOS

El objetivo principal de esta tesis es conocer el efecto que tienen los diferentes grados de riesgo de depredación y los factores ambientales, así como la influencia de los factores individuales sexo, estado reproductor y experiencia adquirida, en las respuestas comportamental y fisiológica de estrés del ratón de campo (*A. sylvaticus*). Además, se pretende analizar cómo varía la ingesta de alimento en función de los niveles medios de metabolitos de testosterona fecal y de las características individuales sexo y estado reproductor.

CAPÍTULO I: el objetivo de este capítulo es comprobar si el ratón de campo es capaz de modular sus respuestas comportamental y fisiológica de estrés ante diferentes grados de riesgo de depredación por zorro rojo (*V. vulpes*). Además, se estudiará la posible variación en ambas respuestas debido a factores individuales como el sexo y el estado reproductor, así como el efecto de la degradación de los compuestos volátiles fecales del depredador a lo largo del tiempo. Por tanto, predecimos que el ratón de campo adaptará las respuestas comportamental y fisiológica de estrés a los diferentes riesgos de depredación percibidos, mostrando unas respuestas más elevadas en la parcela con mayor riesgo de depredación. Además, teniendo en cuenta los requerimientos asociados al sexo y estado reproductor, prevemos una mayor respuesta comportamental y fisiológica en el caso de las hembras y de los individuos reproductores. Finalmente, se prevé una reducción de ambas respuestas con la degradación de los compuestos volátiles fecales del depredador a lo largo del tiempo.

CAPÍTULO II: en este capítulo se examinará el efecto de diferentes grados de riesgo de depredación por zorro rojo sobre la ingesta de alimento en el ratón de campo, teniendo en cuenta los factores individuales sexo y estado reproductor. Además, se estudiará el efecto de la degradación de los compuestos fecales del depredador a lo largo del tiempo y de la experiencia adquirida por el ratón de campo a través de las recapturas. De acuerdo con la hipótesis de asignación del riesgo de depredación (predation risk allocation hypothesis) de Lima y Bednekoff (1999), predecimos una reducción en la ingesta de alimento del ratón de campo a medida que aumente el riesgo de depredación percibido. Así mismo, la hipótesis de partida es un aumento en dicha ingesta con la degradación de los compuestos volátiles en la fase postratamiento y con la experiencia adquirida durante las recapturas, al ser ambos factores indicadores de una reducción en el riesgo de depredación percibido.

CAPÍTULO III: el objetivo de este capítulo es estudiar si factores como los niveles medios de metabolitos de testosterona fecal y las características individuales (sexo y estado reproductor) influyen en la ingesta de alimento del ratón de campo. Nuestra hipótesis de partida es que la ingesta de alimento será mayor en aquellos individuos con menores niveles de testosterona debido al efecto de refugio que puede desencadenar la trampa para aquellos individuos con menor acceso al alimento en condiciones naturales. Así, prevemos que las hembras y los individuos no reproductores presentarán una mayor ingesta, debido a unos mayores requerimientos energéticos en el primer caso y a no encontrarse bajo la competencia de los dominantes reproductores en el segundo.

CAPÍTULO IV: este capítulo fue diseñado para analizar los factores ambientales e individuales que desencadenan la respuesta fisiológica de estrés en el ratón de campo. Para ello se quiere utilizar métodos estadísticos de análisis basados en *Support Vector Machines* (SVMs), evaluando su utilidad en estudios biológicos de este tipo. Así, se predice unos niveles de glucocorticoides mayores en las estaciones y hábitats donde la cobertura arbustiva sea menor. En este sentido, se sugiere que la precipitación abundante, la humedad elevada y las temperaturas cálidas favorecerían la existencia de una buena cobertura vegetal y, por lo tanto, una menor respuesta de estrés fisiológico. Del mismo modo, la luna llena aumentaría los niveles de GCs debido a una mayor exposición de los animales durante las noches de mayor iluminación. Además, se sugiere un incremento en los niveles de GCs en individuos reproductores debido a una mayor demanda energética, y en juveniles, los cuales son frecuentemente desplazados a hábitats de peor calidad con menor cobertura vegetal y mayor riesgo de depredación. Por último, teniendo en cuenta la fuerte relación entre el peso y la condición corporal de los individuos, suponemos un aumento de la respuesta de estrés fisiológico en aquellos individuos con menor peso, debido a que poseen un sistema de defensa más débil frente los depredadores.

THE CONCENTRATION OF FEAR: MICE´S BEHAVIOURAL AND PHYSIOLOGICAL STRESS RESPONSES TO DIFFERENT DEGREES OF PREDATION RISK

Sánchez-González, B., Planillo, A., Navarro-Castilla, Á. and Barja, I. (2017). The concentration of fear: mice´s behavioural and physiological stress responses to different degrees of predation risk. *The Science of Nature – Naturwissenschaften* (under 2nd review).

ABSTRACT

Predation is an unavoidable and dangerous fact in the lifetime of prey animals and some sign of the proximity of a predator may be enough to trigger a response in the prey. We investigated whether different degrees of predation risk by red foxes (*Vulpes vulpes*) evoke behavioural and physiological stress responses in wood mice (*Apodemus sylvaticus*). We examined the variation in mice responses due to individual factors (sex and reproductive status) and related them to the concentration of the volatile compounds from fox faeces over time. In our experiment, we introduced predation cues into four plots based on the following outline: initial odourless phase 0; phase1 in which each plot was subjected to a different concentration treatment (0, 10, 50 and 100% concentration of fresh faeces of red fox) which was renovated each day; and phase 2 just renewing the previous mentioned treatments the first day. Wood mice were live trapped during all three phases and the physiological response was measured non-invasively by analysing faecal corticosterone metabolites (FCM) in freshly collected faeces. Data on the number of captures and stress levels were analysed by Generalized Linear Mixed Models (GLMMs). Males and reproductively active animals were the individuals who entered less into the traps, especially in the highest concentration plot. Variations in FCM concentrations were explained by plot number, the interaction between plot number and treatment phase, and the interaction between the treatment phase and the reproductive status. During phase 1, we detected a significant rise in FCM levels, being greater as predator faecal odour concentration increased. Additionally, reproductively active individuals showed a strong physiological response during both phases 1 and 2. Our results indicated that wood

mice are able to discriminate different degrees of predation risk, which allows them to trigger gradual changes in their behavioural and physiological stress responses.

Keywords: *Apodemus sylvaticus;* chemical recognition; live-trapping; predator avoidance; predation risk; volatile compounds.

INTRODUCTION

Life is not easy for prey species, which daily face a wide range of threats and dangers. Among threatening factors, predation poses a serious risk for small mammals with it additional cost. Predation acts directly on prey ecology by reducing local population density or indirectly by shaping their behaviour, physiology and morphology (Lima and Dill 1990; Apfelbach et al. 2005; Yin et al. 2011; Hegab et al. 2014a; Sánchez-González et al. 2017). Successful predation usually involves at least five steps: detection, identification, approach, subjugation, and consumption (Endler 1986; Sherbrooke 2008; Schmitz et al. 2013). From prey's perspective, avoiding detection by a predator before a possible encounter is the most important step to avoid being eaten. Thus, prey species have developed different mechanisms to prevent primary detection, such as behavioural changes or morphological defences (Lima 1998; Huffard 2006), but also secondary mechanisms when predator is nearby and already detected its prey (e.g. escape behaviours or distasteful flavours) (Huffard 2006; Luttbeg and Trussell 2013).

In mammals, auditory, visual and chemosensory signals play a particularly important role in most intraspecific or interspecific interactions (Gorman 1990), being scent-marking one of the main means of exchanging information at night (Eisenberg and Eisenberg 1981; Macdonald 1985; Kats and Dill 1998; Wyatt 2003). Further, it has been widely demonstrated that carnivore-derived scents from urine (Jorgenson et al. 1978; Barja and de Miguel 2004), faeces (Gese and Ruff 1997; Hutchings and White 2000; Barja et al. 2011; Piñeiro and Barja 2012; Barja and List 2014) and odorous glandular secretions (Albone and Perry 1976; Asa et al. 1985), also induce a broad variety

of defensive responses in small mammals (Dickman and Doncaster 1984; Jędrzejewski et al. 1993; Yin et al. 2011; Hegab et al. 2014a; Navarro-Castilla and Barja 2014b; Tortosa et al. 2015). Thus, chemical cues seem to provide accurate information about when and where the predator may be a threat, allowing prey to adapt their responses (Kusch et al. 2004; Mirza et al. 2006).

According to Apfelbach et al. (2005), the behavioural response seems to be the most common antipredatory defence and it mainly includes modifications in prey daily activities, such as foraging (Verplancke et al. 2010), reproduction (Creel et al. 2007), and use of space (Creel et al. 2005). For instance, besides reducing the time invested on foraging, the presence of a possible predator also changes rodents' foraging behaviour spatially and/or temporally (Fenn and Macdonald 1995; Díaz et al. 2005; Navarro-Castilla et al. 2017a). However, under certain circumstances prey animals will also display a physiological response to predation pressure (Masini et al. 2005; Hegab et al. 2014a). Thereby, when an animal is subjected to the presence of a predator, the endocrine stress response enhances the activation of the hypothalamic-pituitary-adrenocortical (HPA) axis, stimulating the secretion of glucocorticoids (cortisol or corticosterone depending on the species) (Sapolsky et al. 2000; Melmed and Kleinberg 2003). The increase of glucocorticoids has different effects depending on the time of exposure to the stressor. Thereby, short-term stress has been related to an adaptive response for improving prey´s fitness (Wingfield et al. 1998), whereas long-term glucocorticoid secretion may cause some important pathologies (e.g. reproductive failure, endocrine disruption, suppression of the immune system and/or gastrointestinal ulcerations) (Munck et al. 1984; Stewart 2003).

The behavioural and physiological changes in prey's life also involve some costs (Lima and Dill 1990), therefore prey should balance daily activities in relation to the risk of predation perceived in each moment (Lima and Bednekoff 1999; Dielenberg and McGregor 2001; Kavaliers and Choleris 2001). Recent experimental studies have confirmed the threat-sensitive predator avoidance hypothesis described by Helfman (1989). According to this hypothesis, preys are able to use some predator cues characteristics (e.g. differences between fresh/old faeces (Hegab et al. 2014b) to evaluate the situation and take the right decision for surviving a little longer.

OBJECTIVES

The aim of the present study was to test whether wood mice (*A. sylvaticus*) are able to modulate their behavioural and physiological stress responses according to different degrees of predation risk by red fox (*V. vulpes*). We examined the variation in both behavioural and physiological responses due to individual factors, such as sex and mice reproductive status, and related them to concentration and degradation of the volatile compounds from predator faeces over time. We predicted that wood mice would show an avoidance behaviour as well as a physiological response, i.e. an activation of the HPA axis, in response to an increased concentration of predator faecal odour. In addition, we predicted a reduction of these responses with the degradation of volatile faecal compounds over time.

MATERIAL AND METHODS

Live-trapping and data collection

To minimize observer bias, blinded methods were used during the study. Each faecal sample collected was labelled in the field and it was afterwards newly encoded to protect any information regarding its origin. This way, physiological stress levels were analysed in each faecal sample without knowing the identity of the individual.

Live-trapping was performed between February and March 2014. The study area was divided into four plots 35m apart from each other to avoid capturing the same mouse in two different plots. In each plot, we placed 20 Sherman® live traps shaping a 4 x 5 grid with 7 m of distance among them. All traps were covered with vegetation to buffer extreme environmental conditions and they were oriented against the slope in order to allow a correct closing. Total trapping effort was 2400 traps-night (20 traps per grid x 4 plots x 15 nights x 2 trapping sessions).

The experiment was divided into three different phases: preliminary (phase 0), phase 1 and phase 2. Each phase took place during five consecutive days. During phase 0 all plots lacked of experimentally added faecal odour to determine the basal behavioural and physiological responses of wood mice without red fox scent. In phase 1, one plot was used as control with no experimentally added faecal odour and the other three plots were subjected to increasing concentrations of faecal odour: 10% (low concentration plot), 50% (medium concentration plot) and 100% (high concentration plot) to simulate different degrees of predation risk. In this phase, 10 g of faecal material (see section: Simulation of different degrees of

predation risk by faecal odour), were placed outside each trap. Faecal material was renewed every day at sunset to ensure correct odour effectiveness at the maximum activity time of mice, i.e. two or four hours after dusk (Montgomery and Gurnell 1985). Finally, in phase 2 we placed new faecal material just the first day of this phase, in order to evaluate the effect of volatile compounds loss over time. All traps during the three study phases were baited with 4 g of toast corn as a positive stimulus.

Moonlight affects small mammal behaviour (Kaufman and Kaufman 1982; Díaz 1992; Navarro-Castilla and Barja 2014b), so in order to avoid any potential influence of moonlight on our experiments, we selected the nearest days to new moon to carry out our study. Further, Wróbel and Bogdziewicz (2015) recently demonstrated that increased cloud cover enhanced activity of yellow-necked mice (*Apodemus flavicollis*), but this effect tended to be weaker during the full moon. Therefore, we recorded the percentage of cloud cover to control for its possible effect. Traps were checked every 10-12 hours, i.e. at dawn and dusk, to minimize the time that animals were kept.

Each captured individual was identified to species level based on external morphology. Sex and reproductive status of individuals were assessed following Gurnell and Flowerdew (2006). Sex was determined using the anal-genital distance, which is longer in males than in females. In the same way, reproductively active females were classified on basis of the presence of nipples and perforated vaginal membranes; whereas reproductive active males were identified due to the increased size of their testicles that usually descended into the scrotal sac. Individual body weight was measured employing a 100 g

hand-held scale (PESNET, 100 g). In order to have a homogenized sample, we selected only adults and subadults, i.e. those weighing from 13 to over 20 g, as previously described by Lewis (1968a). All captured animals were marked on specific body areas (paws, inner ear area, tail) with harmless waterproof paints (Marking stick DFV, www.divasa-farmavic.com) to identify possible recaptures in each phase and avoid pseudoreplication. All captured animals were immediately released at the same place of capture.

Simulation of different degrees of predation risk by faecal odour

To simulate different degrees of predation risk we used red fox faecal odour, one of the main predators of wood mice (Serafini and Lovari 1993; Padial et al. 2002) because it is well-demonstrated the effectiveness of his faeces to trigger antipredatory responses in small mammals (Dickman and Doncaster 1984; Navarro-Castilla and Barja 2014a; Navarro-Castilla and Barja 2014b). Thus, we collected fresh faeces from a semi captive couple of red foxes (one male and one female) from the Cañada Real Opennature Center (Peralejo, Madrid, Spain). Samples were considered fresh on the characteristics previously defined by Liu et al. (2006) (i.e., those which presented a layer of mucus, a high level of hydration and strong odour). These foxes had a carnivorous diet throughout the experiment similar to what they eat in the wild. Collected faeces were frozen at -20 ºC until used in the experiments to avoid the degradation of volatile compounds (Martín et al. 2010). All red fox collected faeces were thawed and homogenised for one hour and a half.

During the experiment, different degrees of predation risk by red fox were simulated by mixing faecal material with soil and water in three different concentrations, one for each plot: low concentration plot (10% of faecal material), medium concentration plot (50% of faecal material) and high concentration plot (100% of faecal material). Additionally, for the control odourless plot (0% of faecal material), only a mixed of soil and water was prepared. Specific composition of faecal treatments were: control (1000 g of soil from the study area and 250 mL of distillate water), low (100 g of faeces, 900 g of soil from the study area and 250 mL of distillate water), medium (500 g of faeces, 500 g of soil from the study area and 250 mL of distillate water) and high concentration plot (1000 g of faeces and 250 mL of distillate water). Final faecal material was similar in shape and texture to actual faeces in nature in order to avoid a possible bias due to visual cues.

Mice faeces collection and quantification of faecal corticosterone metabolites (FCM).

Measuring faecal glucocorticoid levels is a powerful noninvasive method to assess the physiological response to stress in wild animals (Möstl and Palme 2002; Touma and Palme 2005; Sheriff et al. 2011b; Barja et al. 2012). Fresh faeces from mice were collected each day at sunrise, avoiding the possible influence of circadian rhythms in excretion patterns (Touma et al. 2003), and therefore, in faecal corticosterone measurements. Only faeces without presence of urine were sampled to avoid potential cross contamination effects in our results. Faeces were stored at −20 °C until being processed.

FCM extraction was done following the modified method of Touma et al. (2003). Faeces were dried in a heater (90 ºC, 3 h) and then 0.05 g were weighed and mixed with 500 µl of 80% methanol and 500 µl of phosphate buffer. Then, samples were vortexed by hand for 15 s and in a multivortex for 16 h, followed by 15 min of centrifugation (2500 g). The quantification of FCM levels was done with a commercial corticosterone enzyme immunoassay (DEMEDITEC Diagnostics GmbH, D-24145 Kiel, Germany). The cross-reactivity of the antibodies with other substances according to the manufacturer was 2.4% for 11-Deoxycorticosterone, while cross-reactivity was less than 1% with any other substance (aldosterone, cortisol, progesterone). Since validation of the enzyme immunoassay (parallelism, accuracy and precision tests) is needed (Goymann et al. 1999; Young et al. 2004), we carried out parallelism test with serial dilutions of faecal extracts (1:32, 1:16, 1:8, 1:4, 1:2, 1:1) resulting in a curve parallel to the standard. Accuracy (recovery) was 118.6 ± 31.7 % (n=6). The intra- and inter-assay coefficients of variation for three biological samples were 4.7 % (n = 6) and 8.2 % (n = 3), respectively. In each assay, we used a standard, whose corticosterone metabolite concentration was known. FCM levels are calculated as ng/g dry faeces.

Biological validation carried out by Navarro-Castilla et al. (2017a) has confirmed the suitability of the EIA (enzyme immunoassay) for analysing faecal corticosterone metabolites in wood mouse faecal samples.

Statistical analysis

Both behavioural and physiological stress responses were analysed by Generalized Linear Mixed Models (GLMMs). Behavioural response was analysed using capture frequency as response variable in a model with Poisson error distribution. Regarding the physiological stress response, since weight of individuals has been shown to have a significant influence on FCM in this rodent species (Navarro-Castilla et al. 2014a) we previously corrected FCM by dividing it by the weight of individuals (g) to avoid any possible influence. Later, we used a model with Gaussian error distribution to analyse the concentration of faecal corticosterone metabolites (FCM) (ng/g). For both cases, the explanatory variables were phase (0/1/2), plot (control/low/medium/high), sex (male/female), reproductive status (active/non-active), cloud cover (%), and the interactions between phase and plot, reproductive status and sex, phase and reproductive status, and plot and reproductive status. The potential temporal effect due to the consecutive sampling was controlled by including day as a random factor in the analyses. Assumptions of error distribution and homocedasticity of both analyses were checked in the residuals.

We used likelihood ratio tests (LRT) to estimate the p value of the explanatory variables in the model. We used $\alpha < 0.05$ as the criterion level for significance. Statistical data analyses were done in R 3.3.3 software (R Core Team 2017), using libraries lme4 (Bates et al. 2015) for GLMM and afex (Singmann et al. 2017) for obtaining the *p* values of explanatory variables.

RESULTS

Behavioural response

During the present study, 156 different individuals were captured (see the distribution of captures in Table 1).

Table 1. Distribution of the number of captures of wood mice among sex, reproductive status (RE: reproductively active; NR: Non-reproductively active) and phase.

Number of total individuals captured (N=156)

	Males (N=68)		Females (N=88)	
	NR	RE	NR	RE
Phase 0	21	10	34	10
Phase 1	11	13	20	7
Phase 2	4	9	14	3

Results of the GLMM analysis for capture frequency showed significant effects of reproductive status and the interaction of reproductive status and sex (Table 2).

Table 2. GLMM of the number of captures of wood mice: significant values of explanatory variables estimated by LRT.

Effect	***df***	**Chisq**	***p* value**
Phase	2	5.86	0.05
Plot	3	3.39	0.34
Reproductive status	1	6.16	0.01*
Sex	1	0.01	0.94
Cloudiness	1	2.75	0.10
Phase : plot	6	11.02	0.09
Reproductive status : sex	1	0.15	0.70
Phase : reproductive status	2	9.94	0.007**
Plot : reproductive status	3	5.65	0.13

Looking closer at the effects of the different levels of each analysed factor (Table 3) males were captured less than females (β = -0.635 ± 0.206, p = 0.002), and reproductively active animals less than non-active individuals (β = -0.900 ± 0.433, p = 0.038). However, the interaction between the factors show that number of reproductively active males was significantly high during the whole study (β = 1.108 ± 0.352, p = 0.002). Captures were higher in the highest concentration plot during the three phases (β = 0.989 ± 0.344, p = 0.004) (Fig. 13). However, captures of reproductively active individuals significantly decreased likely due to the avoidance of traps treated with the highest concentration of predator faecal odour (β = -0.976 ± 0.449, p = 0.030) (Fig. 13, dark area).

Table 3. Captures analysis. Summary output of the GLMM that shows relative effect of each level of the analyzed factors.

	Estimate	**Std. Error**	**z value**	**Pr(>|z|)**
(Intercept)	0.257	0.906	0.284	0.777
Phase 1	-0.655	0.800	-0.818	0.413
Phase 2	-1.994	1.063	-1.876	0.061
Low plot	-0.073	0.421	-0.174	0.862
Medium plot	0.184	0.397	0.464	0.642
High plot	0.989	0.344	2.878	0.004
Males	-0.635	0.206	-3.083	0.002
Reproductively active	-0.900	0.433	-2.079	0.038
Cloudiness	-0.001	0.011	-0.129	0.898
Phase 1*Low plot	0.004	0.585	0.007	0.995
Phase 2*Low plot	0.085	0.669	0.127	0.899
Phase 1*Medium plot	-0.098	0.561	-0.174	0.862
Phase 2*Medium plot	0.165	0.625	0.263	0.792
Phase 1*High plot	-0.194	0.483	-0.402	0.688
Phase 2*High plot	-0.573	0.598	-0.959	0.337
Reproductively active *males	1.108	0.352	3.148	0.002
Phase 1* Reproductively active	0.545	0.395	1.380	0.167
Phase 2* Reproductively active	0.547	0.465	1.175	0.240
Low plot* Reproductively active	-0.640	0.529	-1.210	0.226
Medium plot* Reproductively active	-0.776	0.506	-1.532	0.125
High plot* Reproductively active	-0.976	0.449	-2.174	0.030

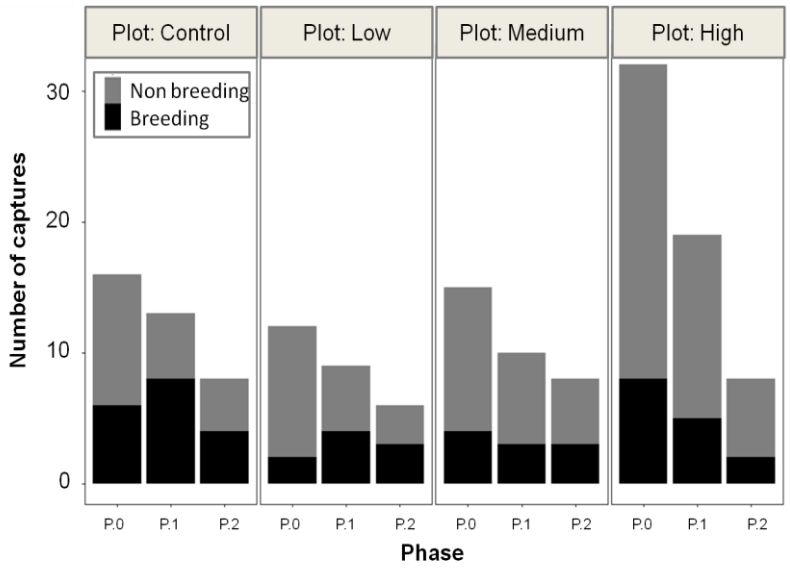


Figure 13. Number of reproductively active and non-active mice captured in each plot during each treatment phase.

Physiological response

The results of the GLMM are shown in Table 4 and Table 5.

Table 4. GLMM of the stress level of wood mice: significant values of explanatory variables estimated by LRT.

Effect	***df***	**Chisq**	***p* value**
Phase	2	4.51	0.1
Plot	3	5.8	0.12
Reproductive status	1	0.22	0.64
Sex	1	9.43	0.002**
Cloudiness	1	0.02	0.9
Phase : plot	6	2.13	0.91
Reproductive status : sex	1	10.23	0.001***
Phase : reproductive status	2	2.43	0.3
Plot : reproductive status	3	5.08	0.17

Table 5. Stress analysis. Summary output of the GLMM that shows relative effect of each level of the analyzed factors.

	Value	**Std.Error**	***df***	***t*-value**	***p*-value**
(Intercept)	4.480	0.309	134	14.484	0.000
Phase 1	-0.589	0.340	10	-1.732	0.114
Phase 2	-0.112	0.442	10	-0.254	0.805
Low plot	-0.751	0.286	134	-2.630	0.010
Medium plot	-0.789	0.245	134	-3.222	0.002
High plot	-0.938	0.219	134	-4.280	0.000
Reproductively active	-0.465	0.272	134	-1.707	0.090
Males	-0.028	0.137	134	-0.205	0.838
Cloudiness	0.005	0.003	10	1.422	0.185
Phase 1*Low plot	0.799	0.402	134	1.984	0.049
Phase 2*Low plot	0.375	0.510	134	0.736	0.463
Phase 1*Medium plot	0.960	0.344	134	2.794	0.006
Phase 2*Medium plot	0.564	0.462	134	1.220	0.225
Phase 1*High plot	0.861	0.325	134	2.653	0.009
Phase 2*High plot	0.573	0.405	134	1.415	0.160
Reproductively active *Males	0.098	0.249	134	0.394	0.694
Phase 1* Reproductively active	0.707	0.258	134	2.737	0.007
Phase 2* Reproductively active	0.696	0.334	134	2.085	0.039
Low plot* Reproductively active	0.060	0.389	134	0.156	0.877
Medium plot* Reproductively active	0.440	0.336	134	1.308	0.193
High plot* Reproductively active	0.592	0.303	134	1.955	0.053

Factors affecting stress response were again sex and reproductive status, although some differences between plots and the interaction of plot and treatment phase can be detected. Thus, although in control plot basal FCM levels were greater than in the rest of plots ($\beta_{\text{Low plot}}$ = -0.751 ± 0.286, p = 0.010; $\beta_{\text{Medium plot}}$ = -0.789 ± 0.245, p = 0.002; $\beta_{\text{High plot}}$ = -0.938 ± 0.219, p < 0.001), during phase 1 a significant rise of FCM was detected, increasing with the increase of predator faecal odour concentration ($\beta_{\text{Low plot}}$ = 0.799 ± 0.402, p = 0.049; $\beta_{\text{Medium plot}}$ = 0.960 ± 0.344, p = 0.006; $\beta_{\text{High plot}}$ = 0.861 ± 0.325, p = 0.009) (Fig. 14). Additionally, reproductively active individuals showed a significant increase in stress levels during both phase 1, with renewal of predator faecal odour (β = 0.707 ± 0.258, p = 0.007), and phase 2, without the renovation (β = 0.696 ±0.334, p = 0.039) (Fig. 15).

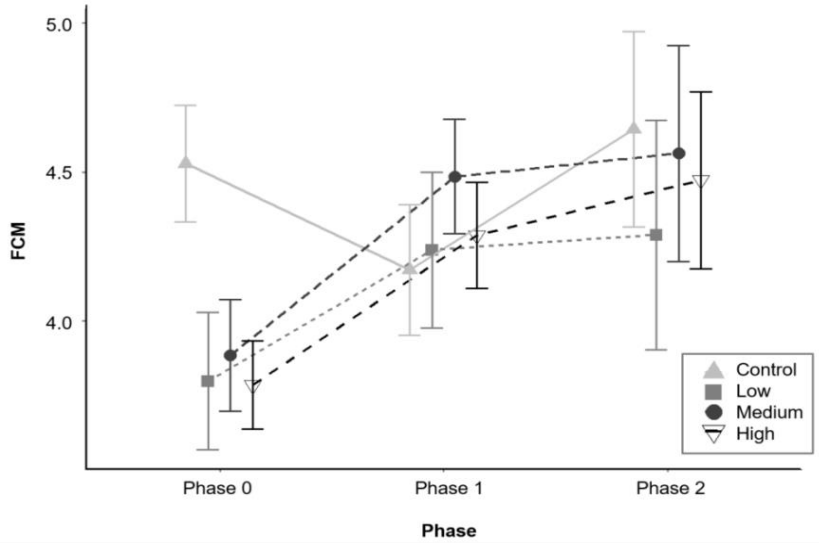


Figure 14. Faecal corticosterone metabolites (FCM) levels in captured mice by plot and treatment phase. Vertical axis is in log scale and whiskers represent the standard errors.

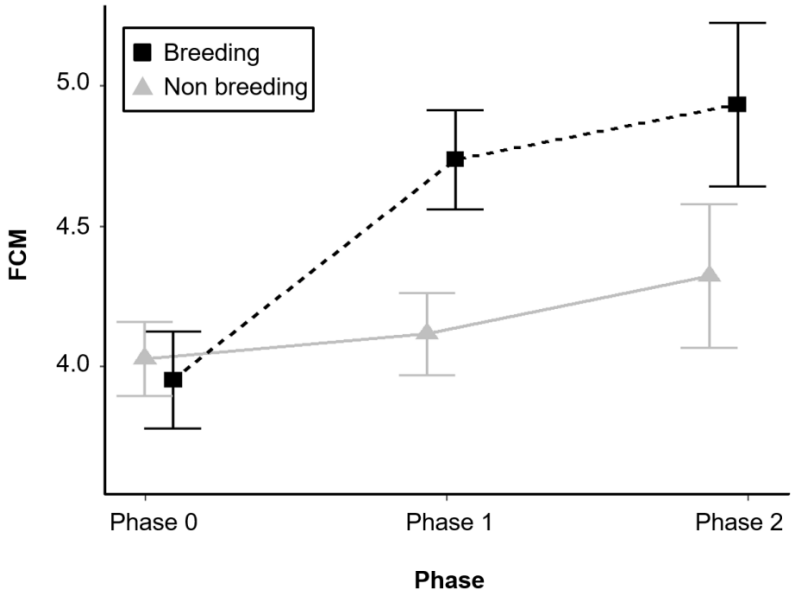


Figure 15. Faecal corticosterone metabolites (FCM) levels detected in captured mice divided by reproductive status and treatment phase. Vertical axis is in log scale and whiskers represent the standard errors.

DISCUSSION

Behavioural response

According to the threat-sensitive predator avoidance hypothesis (Helfman 1989), our study showed evidence that wood mice are able to display a distinctive behavioural response after exposure to different faecal odour concentrations of red fox. This result is also in accordance with other studies where prey species were able to discriminate among different concentrations of chemical predator cues by adjusting the intensity of their responses to match the predation risk perceived (Kusch et al. 2004; Hegab et al. 2014c).

Behavioural changes were influenced by sex and reproductive status. We specifically found an avoidance pattern in reproductively active individuals, both males and females, who clearly avoided traps from the plot with the maximum concentration of faecal odour but entered the rest of traps. This is an interesting result, as during breeding season animals require more resources than in other times of the year (Gittleman and Thompson 1988; Speakman 2008; Dantzer et al. 2010), which implies that they tend to increase foraging to maximize the input of resource to reproduction, even if it means increased exposure to predators. Nevertheless, reproductively active individuals also have to survive long enough to bring up their offspring, and therefore must balance obtaining food with the risk of predation. Our results pointed out that when the concentration exceeds the acceptable limit, 100% of faecal odour in this case, and the trade-off between costs and benefits is broken, wood mice avoid taking risk in order to prioritize breeding and mating, and ensure offspring's success (Montgomery et al. 1991).

Sex was also an important factor and males were less likely to be captured than females. This results could be explained by a reliance of females on energy resources (Montgomery et al. 1991; Penn and Smith 2007), risking more to obtain the food placed inside the traps during the experiment. However, reproductive status could modify this response, since reproductively active males were who entered more into traps. This type of response appears to be related to the fitness costs involved during reproduction in each sex. While males' investment is usually limited to fecundation, females have to ensure the protection of the offspring (Penn and Smith 2007). This essential task of females entails biological costs (e.g. increase mothers'

mortality) which have to be solved without taking more risks than necessary.

Physiological response

The threat-sensitive predator avoidance hypothesis (Helfman 1989) was also corroborated by the physiological results observed. Significant differences found in FCM levels were explained by plot and interaction between plot and treatment phase. Basal FCM concentrations were higher in the control plot than in the rest of the plots, which may be because of some intrinsic factors (e.g. animal transit). However, during phase 1, wood mice exhibited a significant rise of FCM in low, medium and high concentration plots where the predator faecal odour was placed. This antipredatory response due to predation risk has been already found in previous studies performed in different species of mammals (Boonstra et al. 1998; Eilam et al. 1999; Monclús et al. 2006). More interestingly, hormonal secretion seemed to increase with increasing concentration of the faecal odour. Our results showed that mice are able to discriminate among at least three different situations of predation risk (no risk, low risk and medium-high risk), setting the threshold for the antipredator response activation when the concentration reaches 10% and modulating the intensity of this response between low and medium-high risk situations. A positive correlation between the strength of physiological stress response and the concentration of the chemical cue of a predator has been found previously in Brandt's voles (*Lasiopodomys brandtii*) (Hegab et al. 2014c). According to these studies, prey could evaluate chemical cues left by predators triggering the hormonal response only when the perceived signal overcomes a threshold, as posed by the

signal detection theory (Forward et al. 2003; Dupuch et al. 2004). Signal detection theory and threat-sensitive predator avoidance hypothesis complement each other; whilst the first one explains how a prey can set a threshold level of response, the latter deepens in the modulation of this response among different levels perceived. Furthermore, wood mice physiological stress response diminished during the phase 2 which could be explained by the degradation of volatile compounds over time and the loss of sulphurous metabolites by evaporation (Sullivan and Sullivan 1982). Thus, decreased volatile compound concentrations could indicate that a predator is neither present nor close, and therefore, this would be perceived by preys as a less risky situation.

The higher stress hormone levels found in reproductively active individuals in both phases 1 and 2 showed that reproductive status has also a huge influence on the physiological stress response. This result is consistent with other works (Dantzer et al. 2010; Navarro-Castilla et al. 2014b) and supports the important role of glucocorticoids during the reproductive season likely due to pregnancy and lactation in females (Tataranni et al. 1996) as well as the social interaction and competition between males (Navarro-Castilla et al. 2014b). In addition, despite the degradation of faecal volatile compounds over time, reproductively active individuals still showed significant higher FCM levels during phase 2. Thus, reproductive season could be considered as a critic period being individuals more sensitive to predation risk cues.

In summary, we can conclude that wood mice under field conditions seem to be able to discriminate among different degrees of predation risk, modulating their behavioural and physiological stress

responses according to the specific situation. In addition, our results suggest that these antipredatory and physiological responses seem to be modulated by individual factors, highlighting reproductive status. But further research would be necessary to fully understand how individual factors influence prey decision-making process and physiological stress responses under predation risk.

ETHICAL STANDARDS

This research complies with the regulations on the protection of animals used for scientific purposes (Directive 2010/63 / EU of the European Parliament and of the Council of 22 September 2010) and the Spanish legislation (Royal Decree 53/2013). The study had the approval of the Autonomous Community of Madrid (reference number 10/211643.9/13) and favourable reports from both the Ethics Committee of the Autonomous University of Madrid and the Body Enabled (CIS 50-940-A007).

WOOD MICE MODIFY FOOD INTAKE UNDER DIFFERENT DEGREES OF PREDATION RISK: INFLUENCE OF ACQUIRED EXPERIENCE AND DEGRADATION OF PREDATOR´S FAECAL VOLATILE COMPOUNDS

Sánchez-González B., Barja, I. and Navarro-Castilla, Á. (2017) Wood mice modify food intake under different degrees of predation risk: influence of acquired experience and degradation of predator´s faecal volatile compounds. *Chemoecology* 27: 115-122.

ABSTRACT

Behavioural changes in response to predation risk could increase prey survival, but there are associated biological costs. We assessed whether wood mice (*Apodemus sylvaticus*) were able to modulate food intake based on different degrees of predation risk by one of its main predators in the study area, the red fox (*Vulpes vulpes*). Furthermore, we also examined the influence of acquired experience and degradation of the predator's faecal volatile compounds. Wood mice response was analysed by live trapping in four plots during a control phase where all plots were not treated, an experimental phase in which plots were subjected to different concentrations of fresh red fox faeces, and, finally, a post-treatment phase without renewing the faeces. All traps were provided with 4 g of roasted corn, and food intake was calculated based on the amount of bait that remained in each trap. Food intake significantly increased during the experimental phase, especially in the plot treated with the highest concentration of red fox faeces, and also throughout the post-treatment phase. Females and non-breeding individuals showed higher food intake. In addition, there was less food intake in recaptured than new individuals. Our results indicated that wood mice are able to detect different degrees of predation risk and respond accordingly by changing feeding behavioural response; the conflict between food intake and antipredatory behaviour seems to vary with faecal mark strength, which seems to lesson over time. Finally, experience acquired could also play an important role in their daily decision-making process.

Keywords: behavioural changes, biological costs, degrees of predation risk, experience, food intake, volatile compounds.

INTRODUCTION

For prey species, survival in the wild is largely affected by the ability to detect auditory, visual, and chemosensory cues of their predators (Lima and Dill 1990; Kats and Dill 1998; Becker and Gabor 2012; Hettyey et al. 2012; Navarro-Castilla and Barja 2014a). Facing a wide range of existing cues, nocturnal and crepuscular animals, especially mammals, use scent marking as a primary means of exchanging both intra- and interspecific information (Gorman 1990; Torre et al. 2002; Wyatt 2003; Monclús and de Miguel 2003; Brennan and Kendrick 2006). Consequently, territorial marking carried out by most carnivores, which deposit different glandular secretions (Albone and Perry 1976; Asa et al. 1985), urine (Jorgenson et al. 1978) and faeces in conspicuous substrates, becomes a vital information system for nocturnal prey species (Jones and Dayan 2000; Monclús et al. 2005; Fendt 2006; Rouco et al. 2011). A sudden encounter with a predator is usually fatal for prey; consequently, chemical recognition could be a vital advantage, because, in a nocturnal setting, smell is one of the most important senses for anticipating a possible fatal encounter (Apfelbach et al. 2005).

Currently, it is well known that predation strongly influences many aspects of small mammal ecology, both directly by altering population densities and indirectly modifying their daily activities, such as reducing foraging, sociability and grooming, interrupting reproductive activity, or restricting habitat use (Apfelbach et al. 2005; Díaz et al. 2005; Preisser et al. 2005; Creel et al. 2007; Navarro-Castilla and Barja 2014a; Navarro-Castilla and Barja 2014b). These antipredator adaptations often enhance fitness, and also reflect an apparent trade-off between costs (e.g. higher energetic expenditure or

lower mating success) and benefits (e.g. not being injured) that are present during predator–prey interaction (Preisser et al. 2005). Understanding the costs and benefits of avoiding predators' cues is a major challenge in field of behaviour. Many experimental studies have analyzed the variability in an animal's decision making under both indirect (e.g. shelter or moon light) and direct (e.g. predator cues) predation risk (Kotler et al. 1991; Daly et al. 1992; Perea et al. 2011; Navarro-Castilla and Barja 2014b; Busch and Burroni 2015). However, to our knowledge, no study has examined mammalian prey ability to detect and respond to different concentrations of chemical cues (degrees of predation risk) in the wild. However, positive results obtained in aquatic environments (Kesavaraju et al. 2007) showed the importance of this approach. Furthermore, except for the work conducted by Hegab et al. (2014c) under laboratory conditions, little is known about the possible variation in antipredatory response due to the degradation of volatile compounds from predator faeces. This degradation process occurs naturally in the wild, and prey could also consider it as an informative indicator of distance from or proximity to predators.

Behavioural response of small mammals seems to be triggered by a generalized response to volatile sulfurous metabolites present in faeces (Dickman and Doncaster 1984; Dickman 1992) and urine (Nolte et al. 1994) of carnivorous species that are derived from the digestion of animal proteins (Woolhouse and Morgan 1995). However, based on the existence of general compounds from a carnivorous diet, recent studies revealed a close relationship between behavioural response and coevolution of the involved species, which highlighted specific prey–predator recognition (Griffin et al. 2000;

Blumstein et al. 2002; Fendt 2006; McEvoy et al. 2008; Navarro-Castilla and Barja 2014a; Busch and Burroni 2015). Consequently, experience acquired by an individual wood mouse could help inform the prey's decision-making.

OBJECTIVES

Consequently, the aim of the present study was to first analyse the effect of different degrees of predation risk from a natural predator (red fox) on food intake by wood mice, particularly based on breeding condition and sex of individuals. Wood mice are essential prey in the diet of most nocturnal carnivores, such as red foxes, and their ability to recognize and avoid fox faecal odour is well known (Dickman and Doncaster 1984; Navarro-Castilla and Barja 2014a). According to the predation risk allocation hypothesis (Lima and Bednekoff 1999), individuals should increase feeding effort under low-risk situations, and should decrease feeding effort under high predation risk. However, if animals are exposed to long periods of high risk they will be forced to resume feeding to meet their energy requirements. Therefore, we hypothesized that wood mice would change their feeding behavioural response depending on the perception of predation risk. Thus, we expected that mice would decrease food intake in response to an increase in the degree of predation risk previously perceived before going into the trap and while in the trap (likely a safer place), because red fox faecal odour is expected to also be detected inside the trap. Furthermore, we examined the effect of predator faecal volatile compound degradation and the experience acquired by wood mice on food intake, and predicted that food intake

would increase in both cases because of the reduction of perceived predation risk.

MATERIAL AND METHODS

Live-trapping and data collection

Field experiments were conducted between February and March 2014. The response of wood mice to different concentrations of predator faecal odour was studied by live trapping. In the study area, we selected four plots separated by 35 m, and placed 20 Sherman® live traps in each plot in a 4 × 5 grid with 7 m of separation between traps (Fig. 16). All traps were oriented against the slope to avoid interference with closing and problems due to adverse weather conditions.

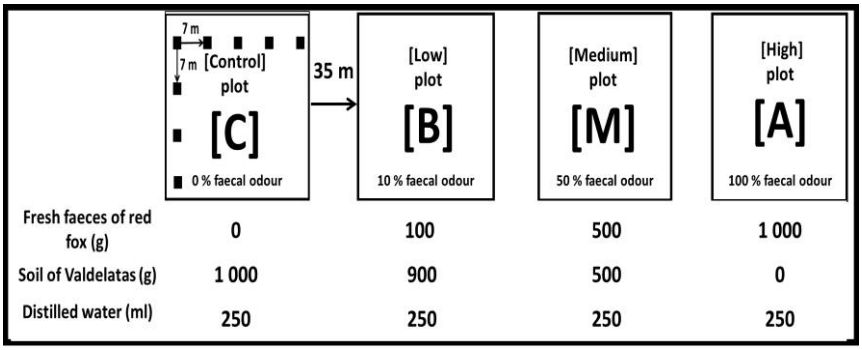


Figure 16. Experimental plots and their associated treatments: C (control, without odour), B (10%, low concentration), M (50%, medium concentration), A (100%, high concentration). Distance between traps within each grid and separation between plots is also shown.

Live trapping was conducted in three phases with different goals: control, experimental, and post-treatment. First, in the control phase, no plots had faecal odour so we could infer the baseline food intake levels. Next, there was an experimental phase, in which one plot acted as a control and each of the other three plots was subjected to one of the three different concentrations of faecal odour, to evaluate the feeding response of wood mice to different degrees of red fox predation risk. In this phase, 10 g of faecal material was placed outside of the trap on one side of the trap entrance to avoid blocking the entry for rodents but close enough to act as a potential risk for each individual trap. The faecal odour was renewed daily at sunset to ensure correct odour effectiveness when mice are more active (i.e. 2 or 4 h after dusk) (Wolton 1983; Montgomery and Gurnell 1985). Finally, in the post-treatment phase (without faecal odour renewal), we evaluated the effect of red fox faecal volatile compound degradation on food intake. All phases lasted five consecutive nights. In the post-treatment phase, we only analysed the first two days for which data were collected because of the fewer number of subsequently captured individuals.

Trapping sessions were conducted during days close to a new moon phase, when wood mice are more active because of reduced predation risk (Kaufman and Kaufman 1982; Díaz 1992; Navarro-Castilla and Barja 2014b), and traps were checked twice daily, at dawn and dusk, to minimize the time that animals were kept. Total trapping effort was 2400 trap nights (20 traps per grid × 4 plots × 15 nights × 2 trapping sessions).

Each captured individual was identified to species by analyzing external morphology, and checked for sex and breeding

condition following the protocols described by Gurnell and Flowerdew (2006). Sex was determined using anal-genital distance, which is shorter in females than males. In breeding adult females, the nipples on the abdomen and thorax are noticeable and the vaginal membrane appears perforated; in breeding adult males, the testicles enlarge quite markedly and usually descend into the scrotal sac. Body weight was measured using a 100-g hand-held scale (PESNET, 100 g), and age class was estimated based on body weight (Lewis 1968a; Behnke et al. 1999): animals that weighed between 0–13 g were considered juveniles, > 13–20 g were considered subadults, and > 30 g were considered adults. All captured animals were marked in non-conspicuous areas with harmless waterproof paints (Marking stick DFV, www.divasa-farmavic.com) to identify possible recaptures and avoid pseudoreplication. All captured animals during each check were released quickly at the same place of capture.

Simulation of different degrees of predation risk by faecal odour

Because wood mice have previously been documented to avoid red fox faeces within an area (Navarro-Castilla and Barja 2014a), the predator treatments were made with fresh red fox faeces collected from a pair of captive animals (one male and one female) reared in the Centro de Naturaleza Opennature Cañada Real (El Escorial, Madrid). We only used fresh faeces (i.e. those which had a layer of mucus, a high level of hydration, and strong odour) (Liu et al. 2006). All faeces were collected at dawn and were frozen at −20 ºC until used in the experiments to prevent degradation of volatile compounds (Martín et al. 2010). In carnivores, volatile compounds vary in relation to

seasonal or individual factors such as sex, age, and breeding condition (Raymer et al. 1984; Andreolini et al. 1987; Hayes et al. 2006). For this reason, all collected red fox faeces were thawed for 1.5 h, and then mixed to obtain a homogeneous mixture. Thus, we ensured a similar initial degree of predation risk to prevent possible bias in our results. Three different predator treatments were established that differed in red fox faecal concentration, and each was applied to a plot: low (10%), medium (50%), and high (100%) concentrations. There was also an odourless control (0%) (see details in Fig.16). Then, 10 g of each faecal treatment was frozen at –20 ºC until the start of field experiments.

Food intake assessment

All traps were baited with 4 g of toasted corn. The remains of unconsumed bait by each captured individual were collected and dried at 80 ºC in a heater for 1 h and then weighed with an electronic balance (C-3000/0.01 g CS, COBOS; precision 0.01 g) to determine the amount of food eaten in each trap by each individual.

Statistical analysis

Food intake under predation risk was analysed by general linear model (GLM) using the amount of food taken (g) corrected by the weight of the animals (g) as the response variable to avoid a possible bias in our results due to differences in consumption based on an individual's weight. The response variable was transformed ($\log_{10}+1$) to produce a normal distribution (Shapiro test) and homogenous variance (Levene test). The independent variables were phase (control/experimental/post-treatment), sex (male/female), breeding

condition (breeding/non-breeding), and recapture (new/recaptured) as fixed factors. Furthermore, because of their biological importance, the interactions between phase and plot, and between phase and recapture were also included in the GLM. Significant factors that influence food intake were analysed by Tukey's honestly significant difference (HSD) post-hoc tests. In addition, because of the significant interaction of phase x plot, ANOVA tests were performed for each plot to analyse individual differences in food intake during the three phases. Finally, we analysed the influence of experience acquired regarding food intake during each phase by performing *T* tests between new individuals and recaptured ones. Results were considered significant at $\alpha < 0.05$. Data are represented as mean ± standard error (SE). Statistical data analysis was performed in SPSS 22.0 for Windows (SPSS Inc, Chicago, IL, U.S.A.).

RESULTS

The total number of wood mice captured was 253 individuals, of which 169 were new and 84 were recaptured. The GLM showed that the significant factors that explained the variation in food intake were phase ($F_{2,173} = 8.044$, $p < 0.0001$), sex ($F_{1,173} = 9.665$, $p = 0.002$), breeding condition ($F_{1,173} = 17.614$, $p < 0.0001$) and interactions between phase and plot ($F_{9,173} = 2.107$, $p = 0.030$) and between phase and recapture ($F_{2,173} = 3.052$, $p = 0.049$) (see more details in Table 6).

Table 6. General linear model results regarding the effect of phase, sex, breeding condition, recapture, and interactions on the amount of food intake in wood mice under different degrees of predation risk.

Factor	***F* value**	***df***	***p* value**
(Intercept)	853.730	1	0.0001
Phase	8.044	2	0.0001
Sex	9.665	1	0.002
Breeding condition	17.614	1	0.0001
Recapture	0.007	1	0.935
Phase x plot	2.107	9	0.030
Phase x recapture	3.052	2	0.049

Wood mice significantly increased food intake during the experimental (Tukey´s HSD, $p = 0.004$) and post-treatment phases (Tukey´s HSD, $p = 0.001$) compared with the control phase. However, differences were not significant between the experimental and post-treatment phases (Tukey´s HSD, $p = 0.505$) (Fig. 17). The significant interaction between phase x plot showed that food intake varied between plots during the three phases (Fig. 18). Individual ANOVA tests performed for each plot revealed that food intake variation between phases was not significant in the control plot ($F_{2,42} = 0.905$, $p = 0.413$), or in the low ($F_{2,30} = 1.775$, $p = 0.189$) and medium-concentration plots ($F_{2,36} = 2.342$, $p = 0.112$). However, food intake significantly varied among phases in the highest concentration plot ($F_{2,61} = 3.529$, $p = 0.036$), and results showed a significant increase in food intake between control and experimental phases (Tukey´s HSD,

p = 0.028) but no differences were found between control and post-treatment phases (Tukey´s HSD, p = 0.599) or between experimental and post-treatment phases (Tukey´s HSD, p = 0.599) (Fig. 18).

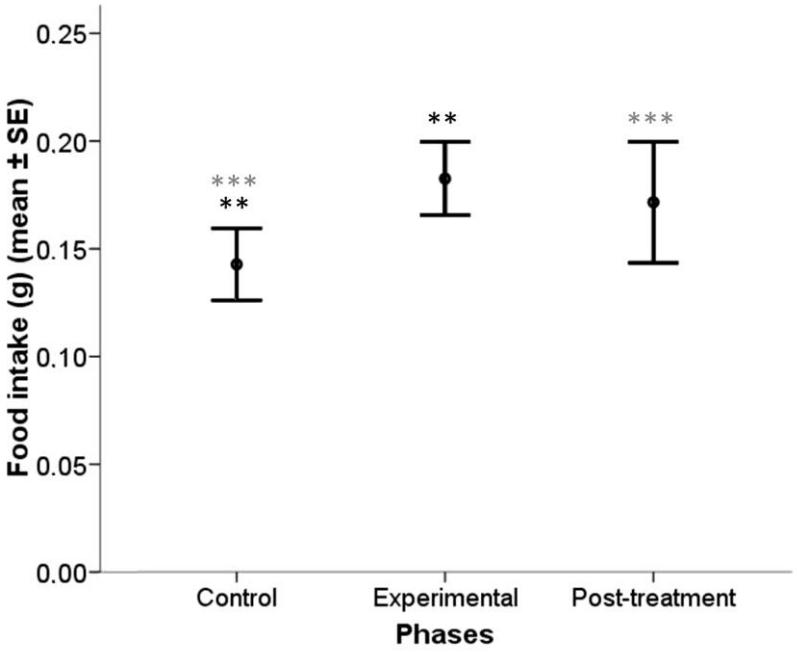


Figure 17. Mean food intake by wood mice during control, experimental, and post-treatment phases. Asterisks indicate significant differences between the analysed groups. * p < 0.05, ** p < 0.01, *** p < 0.001

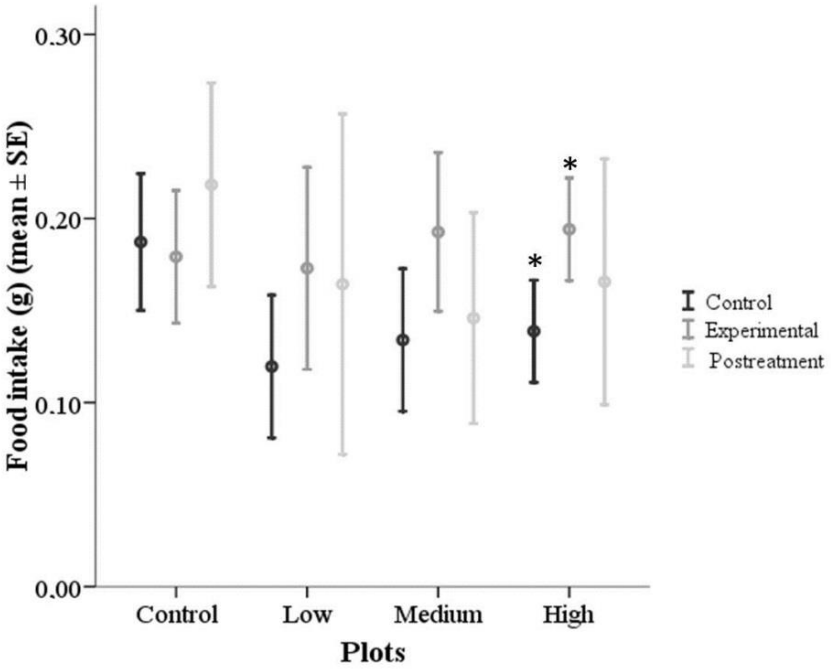


Figure 18. Mean food intake by wood mice in each plot during the three phases. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

For sex, females (0.187 ± 0.007) showed significantly higher food intake than males (0.149 ± 0.010). Furthermore, breeding condition also explained variation found in food intake, because non-breeding individuals (0.189 ± 0.008) showed higher food intake than breeding individuals (0.131 ± 0.007). Regarding the interaction between phase x recapture, food intake did not vary between new and recaptured individuals during the control (*T* test, $p = 0.470$) or post-treatment phases (*T* test, $p = 0.341$). However, captured individuals significantly reduced food intake during the experimental phase compared with new individuals (*T* test, $p = 0.014$) (Fig. 19).

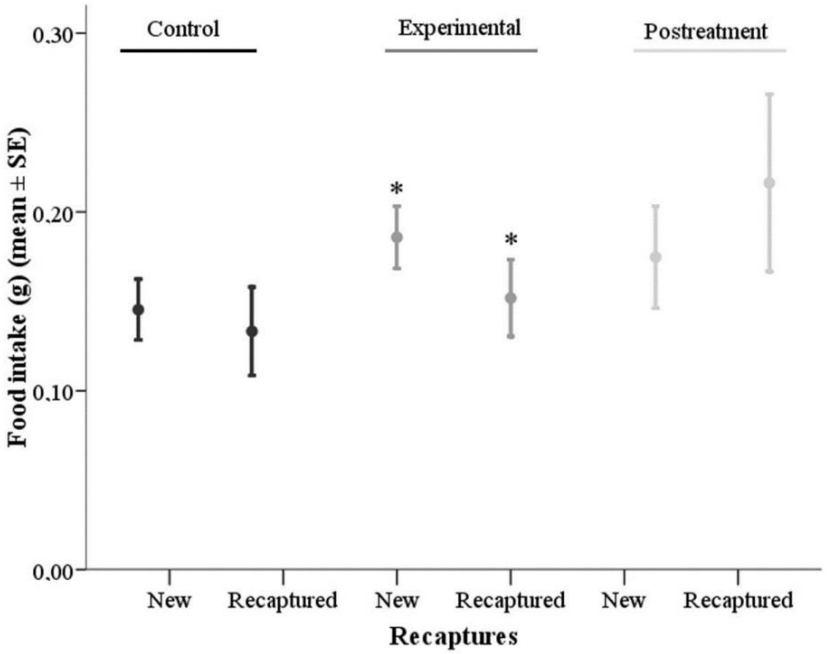


Figure 19. Mean food intake by wood mice in the different phases relative to the recapture factor * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

DISCUSSION

Scent marks left by potential predators have previously been documented to trigger antipredatory responses for many rodents, such as *Lasiopodomys brandtii* (Hegab et al. 2014c), *Microtus agrestis* (Dickman and Doncaster 1984; Bolbroe et al. 2000), *Clethrionomys glareolus* (Dickman and Doncaster 1984) and *A. sylvaticus* (Dickman and Doncaster 1984; Navarro-Castilla and Barja 2014a; Navarro-Castilla and Barja 2014b). However, this is the first study to demonstrate that wood mice are able to distinguish between different concentrations of predator faeces and responding accordingly. Antipredatory behaviour cannot persist indefinitely because of the associated costs (Preisser et al. 2005), so prey have to balance the

distribution of their daily and antipredatory activities suitably throughout the day (Lima and Bednekoff 1999; Sih and McCarthy 2002; Mirza et al. 2006). The present study showed that individuals modified a daily non-defensive routine, food intake, based on perceived predation risk. Thus, the amount of food ingested during the experiments was determined by phase and plot. In the control plot, there was no variation in food intake between the control and experimental phases; however, we found that food intake significantly positively increased with increasing red fox faecal concentration in the highest concentration plot. Thus, food intake accordingly increased as the perceived predation risk was higher in the different plots, which showed an interesting and important trend that should be studied more in depth in future projects. The predation risk allocation hypothesis (Lima and Bednekoff 1999) predicts that an animal under long and frequent high-risk periods should allocate more feeding effort over short low-risk intervals. However, previous experiments on feeding behaviour under predation risk have yielded both reductions and increases in food intake near the scents of a potential predator in many prey organisms, such as *Aplodontia rufa* (Epple et al. 1993), *Arvicola terrestris* (Barreto and Macdonald 1999), *Oryctolagus cuniculus* (Boag and Mlotkiewicz 1994) and *A. sylvaticus* (Navarro-Castilla and Barja 2014a). In our study, during the experimental phase and especially in the high-concentration plot, individuals had to trade-off security and vital necessities in response to the previously perceived degree of predation risk before going into the trap and while being captured, because red fox faecal odour is likely also perceived inside the trap. If we consider traps to be shelters, we suspect that the possible safety effect of the traps would be more important for mice

that experience a higher degree of predation risk. Because the risk allocation hypothesis proposes that animals, during high-risk periods, should allocate more feeding effort in low-risk intervals, the sensation of safety inside traps for mice would be greater when the perceived risk also increases, and this could explain the increased food intake found under the highest predation risk situation compared with the other treatments and control. Similar results after stressful events have been previously observed, and animals increased food intake after being subjected to stressful situations (Wilson and Cantor 1986; Hamilton and Heithaus 2001).

In nature, the risk of encountering a potential predator decreases as time passes, especially in the case of predators that have extensive foraging areas, such as the red fox (Kats and Dill 1998). Thus, we expected that the degradation of volatile compounds simulated in the post-treatment phase of this present study could have been interpreted by prey as a reduction in the probability of being caught by a predator (i.e. a shorter and infrequent high-risk situation rendered it unnecessary to maintain antipredatory behaviour (Lima and Bednekoff 1999). In relation to this, Hegab et al. (2014) recently demonstrated that faeces stored for longer periods lead to a reduction of perceived predation risk, because prey decreased antipredatory responses under laboratory conditions. In our study, increased food intake observed in the experimental phase remained during the post-treatment phase instead of showing a significant reduction. This unexpected result could have been obtained because faecal concentrations 5 days after deposition might not be low enough to significantly reduce prey response. In addition, we found noticeably greater variation during the post-treatment phase than the other

phases, which could be due to reduction in the number of individuals captured in this phase (control phase n= 80; experimental phase n=57 and post-treatment phase n=32), dilution effects on the four different plots, and different reactions caused by individual characteristics (e.g. sex and breeding condition). Nevertheless, concentration of volatile compounds seems to be an important factor in the assessment of predation risk, but further studies are needed to fully understand the antipredatory responses of prey under natural conditions.

The amount of food intake was also affected by breeding condition, because non-breeding individuals showed higher food intake. **A**lthough energy requirements are higher during breeding season (Speakman 2008; Dantzer et al. 2010), breeders have also to take care of their litter and avoid taking risks and prioritizing breeding and mating (Montgomery et al. 1991). However, females also showed higher intake, which could be related probably to greater dependence on resources than males (Montgomery et al. 1991; Penn and Smith 2007). Finally, recapture also had an effect on food intake. Recaptured individuals may have reduced their food intake during the experimental phase in response to a second encounter with predator's odour because of the previous non-harmful experience. Thus, when wood mice came across predator's faecal marks again, the perceived risk might have been lower, because in the preceding catch they were able to obtain food easily without suffering risk (i.e. no predator attack). As a result of previous experience, they seemed to learn that the chemical signals would not be as dangerous as they thought the first time. Furthermore, experience is often treated from a coevolutionary aspect by scientists, without considering the mouse life history perspective. Therefore, although previous predator–prey

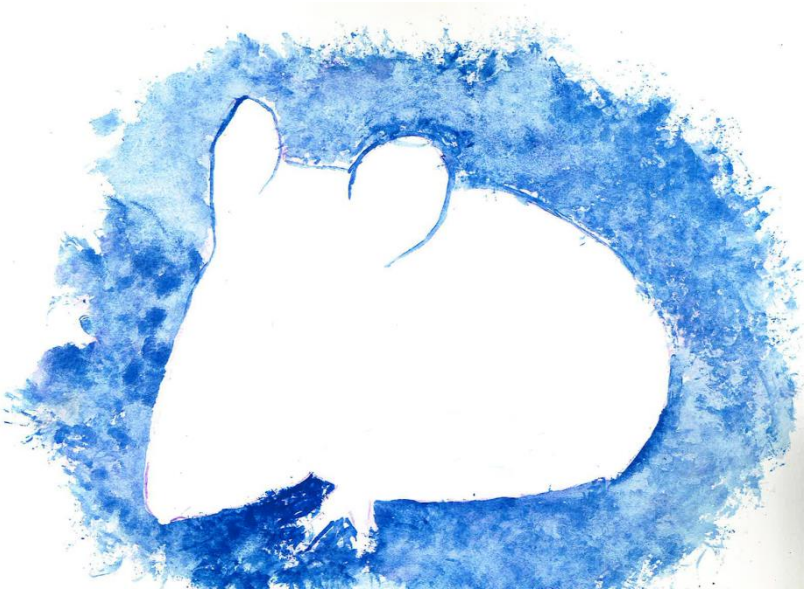
studies have produced mixed results (Dickman 1992; Monclús et al. 2005; McEvoy et al. 2008; Navarro-Castilla and Barja 2014a), it is clear that experience has a role in shaping prey behaviour, and future studies should take this into account to produce more comprehensive results.

In conclusion, red fox faeces seem to be an important cue for wood mice which are able to evaluate different degrees of predation risk based on altering food intake. Thus, wood mice increased food intake as a behavioural strategy in response to long-frequent high-risk situations, and consistently did so over time even when the threat was gone. In addition, our results suggested that this antipredatory response seems to be modulated by individual factors, such as sex, breeding condition, and life experience. Consequently, prey behavioural responses to predation risk cannot be generalised, and further research is necessary to elucidate how prey species cope with each risky situation.

ETHICS STATEMENT

This research complies with the regulations on the protection of animals used for scientific purposes (Directive 2010/63 / EU of the European Parliament and of the Council of 22 September 2010 and the Spanish legislation (Royal Decree 53/2013). The study had the approval of the Autonomous Community of Madrid (reference number 10/211643.9/13) and favourable reports from both the Ethics Committee of the Autonomous University of Madrid and the Body Enabled (CIS 50-940-A007).

FAECAL TESTOSTERONE METABOLITE LEVELS AND INDIVIDUAL CHARACTERISTICS MODIFY FOOD INTAKE IN WOOD MICE (*APODEMUS SYLVATICUS*)



Sánchez-González, B., Barja, I. and Navarro-Castilla, Á. Faecal testosterone levels and individual characteristics modify food intake in wood mice (*Apodemus sylvaticus*).

ABSTRACT

Food intake in animals like micromammals can be influenced both by individual characteristics and physiological traits. In this study we evaluated whether or not factors such as mean faecal testosterone levels and individual characteristics (sex and reproductive status) influenced food intake in wood mouse (*Apodemus sylvaticus* Linnaeus, 1758). To do this, live trapping were carried out in February and March of 2014 and 2016, in Monte de Valdelatas (Madrid). Within the study area four plots were established, separated by 35 m, in which 20 Sherman traps were set, with 7 m spacing. Sampling was performed over 10 consecutive days. All the traps were baited with 4 g of toasted corn. The amount of food ingested was calculated from the remaining food found in the traps. The testosterone metabolites in the fresh faeces from each captured individual were quantified through a competitive enzyme immunoassay using the ELISA technique. The variation in food intake by the wood mice was explained through faecal testosterone levels, sex, and reproductive status. Females ingested the most, apparently due to greater energy requirements, together with non-reproductively active mice. These latter individuals have a low social status and this led to them ingesting a larger quantity in the traps as they had direct access to food with no competition.

Keywords: competition, faecal testosterone concentration, food intake, small mammals, social status.

INTRODUCTION

Steroid hormones play a key role in the life of numerous vertebrate species. In particular, the levels of testosterone, a hormone from the androgen group, directly affect the morphological, behavioural, and physiological aspects of many animal species (Ketterson and Nolan 1992; Dunlap and Schall 1995; Hughes and Randolph 2001; Amstislavskaya and Popova 2004; Goymann et al. 2004; Jawor et al. 2007). These effects include its relationship with the social status of an individual, due to the significant implications this has for the survival of a population. It has been observed that males with increased levels of testosterone attain a higher social status, exhibiting greater dominance than males with lower levels, which play the role of subordinates (Mazur and Lamb 1980; Greenberg and Crews 1990; Holekamp and Smale 1998). This increased dominance affects inter- and intrasexual selection and, therefore, the reproductive success of an individual (Enstrom et al. 1997). Thus, during the breeding season, when intraspecific competition among males increases, aggressive behaviour linked to the defence of territory, females, and offspring is also elevated due to higher levels of testosterone in that period (Salvador et al. 1996; Briganti et al. 1999; McGlothlin et al. 2007; McGlothlin et al. 2008). This means dominant individuals have a greater probability of obtaining the desired resource (females, food, and territory, among other things), and preserving this over time (Gray 1971; Trainor et al. 2004). The direct implication of this fact is an increase in the number of females they mate with, the protection of these ensuring the survival of the offspring (Enstrom et al. 1997). The production of good quality sexual signals, for the purposes of intraspecific sexual ornamentation, is another equally important factor

when it comes to ensuring reproductive success. However, the production of these is also modified according to testosterone levels, and is even inhibited when the levels of this hormone are excessively low (Cox et al. 2005). Behaviour when looking for food and greater locomotive activity are also influenced by testosterone, these activities increasing with augmented hormone levels (Moore and Marler 1987; Hughes and Randolph 2001).

The endocrine system is, therefore, the immediate link between male morphology, behavioural changes associated with defending the territory and maintenance of social status, and reproductive success, among other things (Mills et al. 2009). In addition, some studies have shown that testosterone levels have implications for immunological functions, suggesting that testosterone is one of the physiological factors that regulates reproduction and survival compensation (Charles 1984; Dunlap and Schall 1995; Saino et al. 1995; Peters 2000; Grear et al. 2009).

Steroid hormone production in mammals, in this case testosterone, is controlled by the hypothalamic–pituitary–gonadal axis (HPG axis) (Arce et al. 2006) (Fig. 20). The hypothalamus secretes a gonadotropin-releasing hormone (GnRH) that stimulates the adenohypophysis to produce two types of hormones: follicle stimulating hormone (FSH) and luteinising hormone (LH) (Arce et al. 2006). Luteinising hormone regulates and stimulates the biosynthesis of testosterone in the Leydig cells (Brooks 1975; Arce et al. 2006), located in the testicular interstitial tissue. Follicle stimulating hormone stimulates spermatogenesis by acting on the Sertoli cells, located in the seminiferous tubules. GnRH secretion is regulated by a negative feedback system that helps maintain hormonal stability (Arce et al.

2006) (Fig. 20). In addition, activating the physiological stress response can also act as an inhibitory system, since the functioning of the hypothalamus-pituitary-adrenal axis inhibits GnRH production, decreasing its secretion and, therefore, reducing the levels of FSH and LH in the adenohypophysis, causing it to decrease testosterone secretion (Handa et al.1994).

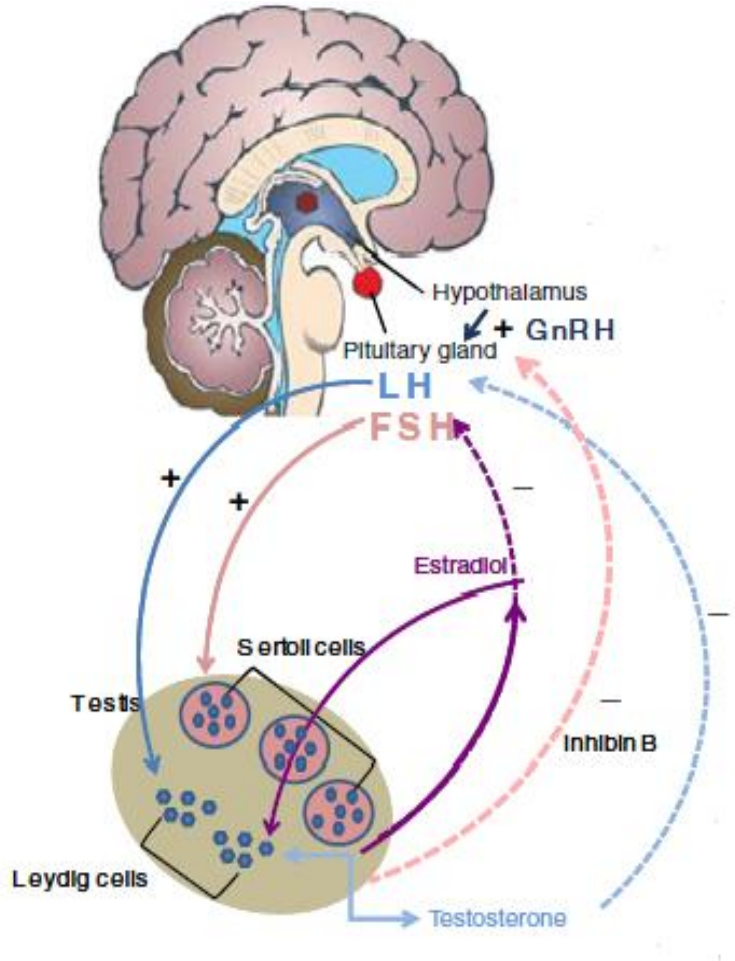


Figure 20. Cycle of the hypothalamic–pituitary–gonadal axis (HPG axis) responsible for the production of testosterone (Andersen et al. 2011).

OBJETIVES

The main objective of the present study was to understand how food intake in the wood mouse (*A. sylvaticus*) varies based on faecal testosterone metabolite levels and individual factors like sex and reproductive status. It was predicted that there would be greater food intake in those individuals with lower testosterone levels because there would be less competition for food once they had been captured. Our second prediction was that females would ingest more food than males due to their greater energy requirements. Finally, we hypothesised that non-reproductively active individuals, which normally play the role of subordinates in a population, would exhibit greater food intake during their capture due to not having to compete with dominant animals under these conditions.

MATERIALS AND METHODS

Live-trapping and data collection

The study was carried out in February and March of 2014 and 2016, in Monte de Valdelatas (Madrid). To study the variation in food intake based on faecal testosterone metabolite levels and individual characteristics (sex and reproductive status), two live-trapping campaigns were undertaken. This technique is widely used as a non-invasive sampling method in micromammal studies as it is highly effective (Torre et al. 2004). Each campaign lasted 10 consecutive days. In the three days prior to each session, preliminary sampling was carried out in the study area to verify whether wood mouse abundance was adequate for the experiment. Traps were placed in four 588 m^2

plots with similar characteristics. These were spaced 35 m apart to reduce the probability of recapturing the same individuals in different plots, as this exceeds the distances traversed by the wood mouse (Blanco 1998). In each plot 20 Sherman® live capture traps were set, in a 4 x 5 grid, spaced 7 m apart from one another (Gurnell and Flowerdew 2006) (see Fig. 16). The traps were placed in areas with a high degree of plant cover, where more wood mice were present as these were the zones with the greatest energy resources and most defence from predators (Bowers 1988; Kotler and Brown 1988; Torre et al. 2002). All the traps were oriented against the slope, reducing the likelihood of interference when closing. Each trap was reviewed at dawn and dusk, coinciding with the nocturnal activity of the wood mouse (Wolton 1983) and also minimising the time the animals were inside the traps. Total trapping effort was 3200 trap nights (20 traps per grid × 4 plots × 10 nights × 2 trapping sessions × 2 years).

Each individual captured in the traps was identified to species level. This was done using characteristics of their external morphology: body size (length of ears and tail), coat colouration, contrast between the dorsal and ventral zones, and the presence of bulging eyes. Once the species had been identified, the individuals were introduced into a net to make it easier to handle them. Each individual was then weighed on a scale (PESNET, 100 g). In accordance with Gurnell and Flowerdew (2006), the sex was determined considering the anal-genital distance, this distance being greater in males than females. The reproductive status of each captured animal was determined though a physical examination, considering males with descended testicles and females with

prominent mammary glands on the thorax and/or a perforated vagina to be reproductively active (Gurnell and Flowerdew 2006).

To identify the captured specimens and avoid replicating data in the statistical analyses, the individuals were marked with various coloured harmless paints (DFV Marking stick, www.divasa-farmavic.com) on different parts of the body. All the captured animals were handled for as little time as possible and released where they had been trapped.

Food intake assessment

To find out how food intake varies in the wood mouse, each trap was baited with 4 g of toasted corn. The bait was weighed in the laboratory using a precision electronic scale (C-3000/0.01 g CS, COBOS; accurate to 0.01 g). Whenever an individual was captured, the unconsumed food was collected and placed into suitably labelled individual bags that were stored in the laboratory for further analysis. In order to discover how much food had been ingested by the animals the trap, each collected food sample was dried in a heater at 80ºC for 1 hour, and then weighed. All the traps were disassembled after each capture for cleaning and resetting.

Faeces collection and quantification of faecal testosterone metabolites

Fresh wood mice faeces were collected at dawn, in order to minimise the degradation of the steroid hormones by microorganisms (Möstl et al. 2005; Barja et al. 2012). Any faeces contaminated with urine were rejected to avoid a bias in the results. The faecal samples were conserved at -20 ºC until their later analysis in the laboratory.

To analyse the faecal testosterone metabolite (FTM) concentrations, the water content of the faeces that had been preserved at -20 ºC was eliminated in a heater (at 90 ºC for 3 h). 0.05 g of the sample was then weighed out and mixed with 500 mL of 80% methanol and 500 mL of phosphate buffer. The samples were mixed in a manual vortex for 15 seconds and an orbital agitator for 16 hours. They were subsequently centrifuged for 15 minutes at 2000 rpm and the supernatant of these samples, the faecal extracts, was stored at -20 ºC until the hormonal analysis. In order to discover the testosterone concentration in the faecal samples a competitive enzyme immunoassay (EIA) was undertaken using the ELISA technique. The later assessment of FTM concentration was carried out using a spectrophotometer. The EIA of wood mouse faeces was validated for this species following the methods described by Bamberg et al. (2001) and Morrow et al. (2002). Since validation of the enzyme immunoassay (parallelism, accuracy and precision tests) is needed (Goymann et al. 1999; Young et al. 2004), it was carried out parallelism test with serial dilutions of faecal extracts (1:32, 1:16, 1:8, 1:4, 1:2, 1:1) resulting in a curve parallel to the standard. Accuracy (recovery) was 104.4 ± 6.9 % (n=12). The intra- and inter-assay coefficients of variation for three biological samples were 3.6 % (n = 3) and 7.1 % (n = 3), respectively. FTM levels are expressed as ng/g dry faeces.

Statistical analysis

To statistically analyse the data, the Kolmogórov-Smirnov test was run on the quantitative variables to determine whether the data fit a normal distribution, and the Levene test was used to find out if there

was homogeneity of variance. The variables food intake, abundance of wood mice per plot, and mean FTM levels were transformed (log_{10}+1) to fit a normal distribution.

To analyse food intake in function of the studied variables a general linear model (GLM) was run, using food intake (g) corrected for the weight of each individual (g) as a dependent variable. In the model, sex (male/female) and reproductive status (reproductively active/non-reproductively active) were used as predictor variables, and mean faecal testosterone metabolite level (ng/g) and wood mouse abundance in each plot were used as covariables. In addition, the sex*reproductive state interaction was assessed because of its biological importance.

A Pearson correlation analysis was also performed to measure the interdependence between food intake corrected for weight (g) and faecal testosterone metabolite levels (ng/g).

The significance level estimated to reject the null hypothesis was $p<0.05$. The software used for the statistical analyses of the data was SPSS 23.0 for Windows (SPSS Inc., Chicago, IL, U.S.A.). The data is presented as the mean ± SE.

RESULTS

A total of 130 wood mice individuals were captured: 61 males and 69 females.

The GLM indicates that the variation in food intake by the wood mice can be explained by reproductive status, sex, and FTM levels (Table 7). The variable abundance of wood mice in each plot and the interaction between sex and reproductive status were not significant (Table 7).

Table 7. General linear model (GLM) results on the effect of sex, reproductive status, wood mouse abundance, FTM levels, and the interaction of sex*reproductive status on the amount of food intake (g) in wood mice.

Factors	***F* value**	***df***	***p* value**
Sex	4.309	1	0.040
Reproductive status	10.566	1	0.001
Testosterone	4.462	1	0.037
Abundance	0.349	1	0.555
Reproductive status*sex	0.200	1	0.655

Mean food intake levels in the wood mice were significantly higher in females (0.166 ± 0.008 g) than in males (0.133 ± 0.009 g) (Fig. 21) (F = 4.309, df = 1, p = 0.040 , n = 130).

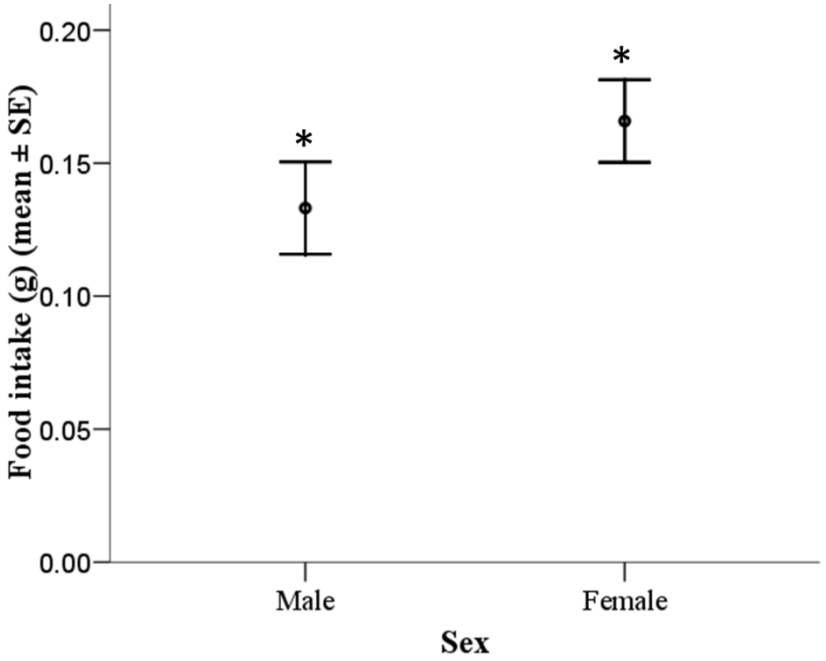


Figure 21. Mean food intake (g) of wood mice by sex.* p < 0.05, ** p < 0.01, *** p < 0.001

In relation to reproductive status, food intake was greater in non-reproductively active individuals (0.165 ± 0.008 g) compared with reproductively active individuals (0.121 ± 0.008 g) (Fig. 22) (F = 10.566, df = 1, p = 0.001, n=130).

It was also observed that as faecal testosterone levels increased, less food was ingested by the individuals (Correlación de Pearson: r = -0.177, p = 0.044, n = 130) (Fig. 23).

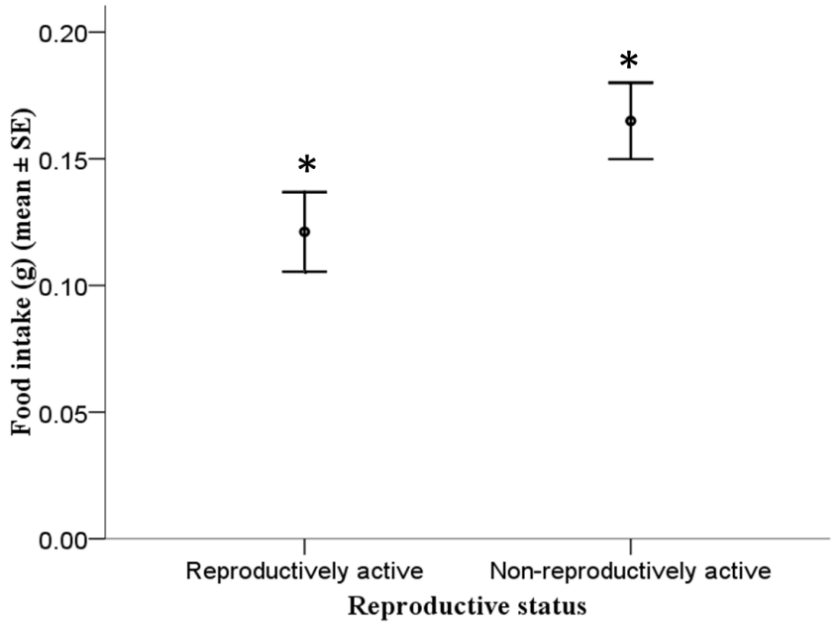


Figure 22. Mean food intake (g) in wood mice by reproductive status. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

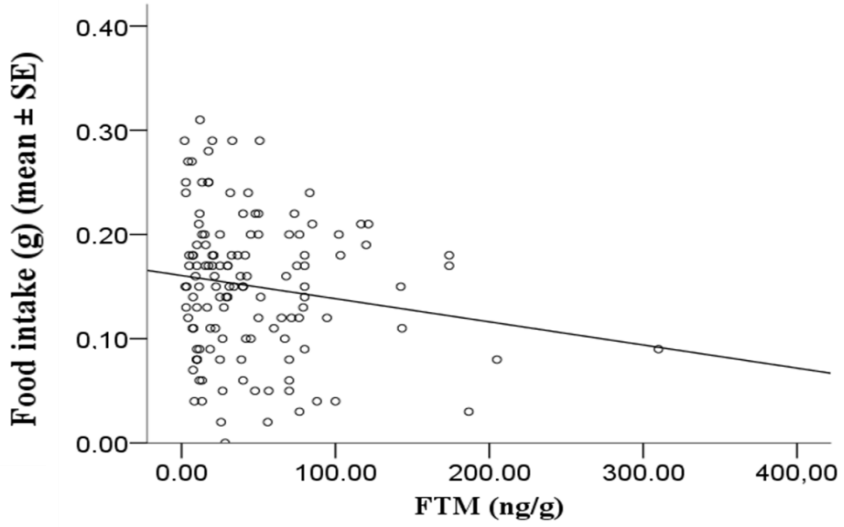


Figure 23. Food intake levels correlated with faecal testosterone metabolite levels.

DISCUSSION

Food intake variation was explained by the individual factors sex and reproductive status, as well as by mean levels of faecal testosterone metabolites. The results showed a higher food intake in females than in males, coinciding with the results observed in previous studies performed in the same study area (Sánchez-González et al. 2017). These results can be explained by the differences found in energy demand between sexes. In general, females present a greater dependence of the resources than males, preferring those areas that have greater food availability, whereas males prefer those with the highest number of reproductive females (Montgomery et al. 1991). This intersexual difference increases greatly during the reproductive period due to the greater energetic cost of reproduction for females, which have a much more active role than males in gestation and care of offspring (Montgomery et al. 1991; Penn y Smith 2007).

On the other hand, food intake also varied according to the reproductive status. Thus, non-reproductively active individuals showed a higher food intake. This result seems to be due to the fact that, in general, non-breeding individuals are subordinate and they have to compete for resources with the reproductively active individuals. The relationship among social status, reproductive status and weight has been previously studied, resulting that the higher weight individuals have a higher dominance status (Alcántara y Díaz 1996), being adults and generally reproductively active individuals. Generally, subordinate individuals are ousted towards lower quality territories in terms of trophic resources, plant cover and predation risk, among others (Wauters y Dhondt 1989; Bondrup-Nielsen y Ims 1990; Dickman et al. 1991). As a consequence of this worse social status, we

could assume an effect of the Sherman traps acting as a refuge for wood mice (Sánchez-González et al. 2017). This effect could explain the higher food intake observed by non-breeding individuals, as, inside traps, they are not under the competition of breeders, thus having a free access to food resource. In addition, it may also be due to the fact that breeding individuals, in spite of requiring a higher energy intake during breeding season (Speakman 2008; Dantzer et al. 2010), give priority to reproduction and the survival of the offspring by reducing those daily activities that may suppose a danger, such as foraging (Montgomery et al. 1991).

Finally, we found a negative correlation between food intake and faecal testosterone metabolites levels. Individuals with lower testosterone hormone levels had a higher food intake than those with a higher production of this hormone. According to Mills et al. (2009), a greater production of testosterone reflects a greater social status, and therefore a greater availability of trophic resources and breeding females, which in turn ensures a better body condition and greater reproductive success. In this context, the subordinate individuals inside the traps would be under a shelter effect, since it is a place where they have no competition for food with more dominant individuals.

ETHICAL STANDARDS

This research complies with the regulations on the protection of animals used for scientific purposes (Directive 2010/63 / EU of the European Parliament and of the Council of 22 September 2010 and the Spanish legislation (Royal Decree 53/2013). The study had the approval of the Autonomous Community of Madrid (reference

number 10/211643.9/13) and favourable reports from both the Ethics Committee of the Autonomous University of Madrid and the Body Enabled (CIS 50-940-A007).

SUPPORT VECTOR MACHINES FOR PREDICTING PHYSIOLOGICAL STRESS IN RELATION TO ENVIRONMENTAL FACTORS IN WOOD MICE (*APODEMUS SYLVATICUS*)

Sánchez-González, B., Barja, I., Piñeiro, A., Hernández-González, M.C., Silván, G., Illera, J.C. and Latorre, R. (2017). Support vector machines for predicting physiological stress in relation to environmental factors in wood mice (*Apodemus sylvaticus*). *Scientific Reports* (under 2nd review).

ABSTRACT

We asses different environmental and individual factors to identify which of them could be the main factors explaining the stress response in Wood mice, *Apodemus sylvaticus*. This adaptive mechanism is crucial for prey species survival. We analyse wild mice faecal samples collected seasonally during three consecutive years. We use support vector machines to predict the faecal glucocorticoid metabolite (FCM) concentration, which is related to physiological stress response, as a function of different stressors. These analysis tools demonstrate to be reliable to infer knowledge from small-sized and high-dimensional datasets. Our results corroborate that the stress response is a complex process where multiple factors can simultaneously participate in a context-dependent manner, i.e., the role of each potential stressor may vary in time depending on other stressors. However, the combination of air humidity, temperature and body weight allows predicting the FCM level fluctuation of 98% of our mice faecal samples. We conclude that significance of humidity and temperature could be mainly related to the existence of a good vegetation cover, influencing the stress response because of food availability and predation risk perception. Weight seems to be related to the stress produced by reproduction and other intraspecific relationships like social dominance and territorial behaviour.

Keywords: environmental factors; humidity; physiological stress response; Support Vector Machines; temperature.

INTRODUCTION

Life forms are influenced by an ever-changing environment. Consequently, animals have developed a wide variety of physiological, behavioural and morphological adaptations to endure harsh conditions and threats (Wingfield et al. 1997; Willmer et al. 2009). Among this extensive array of responses, physiological changes are certainly important, as they increase the energy available for individuals to cope with environmental stressors (Selye 1960).

Physiological stress response plays a key role in the adaptability of animals to changes in the environment, as well as being a decisive factor in the stability of homeostasis (Möstl and Palme 2002). This endocrine response enhances the activation of the hypothalamic-pituitary-adrenocortical (HPA) axis, stimulating the secretion of glucocorticoids (GCs) in the adrenal cortex to overcome adverse situations more effectively (Sapolsky et al. 2000; Melmed and Kleinberg 2003). The short-term release of GCs is an adaptive response that redirects energy from non-vital activities toward survival (Sapolsky et al. 2000; Wingfield and Romero 2001). However, chronically elevated GC levels due to prolonged exposure to stressors may induce deleterious effects such as reproductive disruption, suppression of the immune and endocrine function or inhibition of growth, which can lead to a survival rate and fitness reduction (Sapolsky et al. 2000; Möstl and Palme 2002; Romero 2004). GCs have been used as indicators of animal stress in several species including small mammals (e.g., see Refs. Harper and Austad 2001; Fletcher and Boonstra 2006a; Götz and Stefanski 2007; Navarro-Castilla and Barja 2014b; Navarro-Castilla et al. 2014b). Particularly in wildlife studies, faecal cortisol/corticosterone

metabolites (FCM) have been widely used as a suitable non-invasive measure of the GC levels in order to evaluate responses during stressful circumstances (Barja et al. 2012), reflecting free GC in plasma and yielding an accurate profile of the adrenocortical activity (Wingfield et al. 1997; Goymann et al. 1999; Touma and Palme 2005)

Multiple factors, such as human disturbances (Barja et al. 2007; Piñeiro et al. 2012; Navarro-Castilla et al. 2014a; Navarro-Castilla et al. 2014b), reproduction (Barja et al. 2011; Escribano-Avila et al. 2013), social dominance (Creel 2001; Barja et al. 2008) or habitat type and seasonality (Wingfield et al. 1997; Hik et al. 2001; Sheriff et al. 2012), have been reported to influence the mammals physiological stress response in different ways. Rodent populations often show important seasonal and inter-annual fluctuations, decreasing during seasons with unfavorable climatic conditions and increasing during favourable periods (Montgomery 1989; Fernández et al. 1996; Torre et al. 2002). Seasonal climatic variations determine food availability and plant abundance, which is crucial for predator avoidance (Tew and Macdonald 1993; Ouin et al. 2000). In general, small mammals usually show a strong preference for habitats with a highly dense vegetation cover (Rosalino et al. 2011a; Rosalino et al. 2011b). This increases the survival probability during unfavorable climatic periods and constitutes an effective anti-predator strategy that reduces the exposure to threats (Bowers 1988; Kotler and Brown 1988; Jedrzejewska and Jedrzejewski 1990; Tew and Macdonald 1993; Ouin et al. 2000; Navarro-Castilla et al. 2017b). Moonlight can make animals more visible to predators and has a demonstrated effect on small mammals (Díaz 1992; Navarro-Castilla and Barja 2014b). Predation risk does not only induce behavioural changes, but also

increases GC secretion as part of the stress response (Sánchez-González et al. 2017). These vital behavioural and physiological changes have some costs (Lima y Dill 1990) and preys should balance daily activities in relation to the predation risk perceived in each moment (Lima y Bednekoff 1999; Dielenberg and McGregor 2001; Kavaliers and Choleris 2001). In the particular case of wood mice (*A. sylvaticus*), animals show predilection for evergreen forest and Mediterranean shrub habitats, which provide diverse trophic resources and a large number of shelters due to an abundant vegetation cover (Torre et al. 2002). Individual characteristics of each animal, such as body condition, sex or breeding condition, can determine significant aspects of the mice biology, e.g., home range (Dickman et al. 1991; Tew and Macdonald 1994; Corp et al. 1997) or stress level (Touma et al. 2003; Navarro-Castilla et al. 2014b; Navarro-Castilla and Barja 2014b). At this regard, a relevant result for our study is that the basal GC level of males and females is different due to differences in the GC metabolism and excretion rate (Touma et al. 2003). According to Handa et al. (1994), testosterone acts as a HPA axis inhibitor, regulating the reproductive function in males, while estrogen promotes GC release increasing the amount of energy available for the breeding season in females.

OBJECTIVES

In this paper, we assess the effect of different environmental and individual factors capable of triggering the physiological stress response of wild wood mice. Our goal is to identify which of these factors could most significantly affect the GC level, and hence be the principal stressors in *A. sylvaticus*. Taking into account the above-

mentioned premises and the properties of the study area, we analyse the following factors: year, month, season, rainfall, temperature, relative air humidity, habitat, moon phase, sex, breeding condition and body weight. We consider year, season and month to be time periods grouping a set of factors that could produce together a characteristic effect in the stress response (e.g., favourable/unfavorable weather conditions or reproductive period). We hypothesize that abundant rainfall, high humidity and warm temperatures would favour increasing food availability and, therefore, a smaller physiological stress response. We argue that a higher predation risk perception would elevate the animals' stress response during adverse climatic periods and in habitats with a reduced vegetation cover. Similarly, full moon would elevate GC levels due to a higher exposure of the animals during brightly nights. We also hypothesize an increasing GC levels in breeders, due to a greater energy demand, and in juveniles, which are frequently displaced to poorer quality home ranges with less vegetation cover and higher predation risk. Finally, taking into account the strong correlation between weight and body condition, we suppose more stress in smallest and weakest animals due to intraspecific competitions and a poorer defense system against predators.

MATERIALS AND METHODS

Live-trapping and data collection

The project had the permission of Montes do Invernadeiro Natural Park (Spain) and regional government of Galicia. All procedures were performed in accordance with the ethical standards of the

institution (Universidad Autónoma de Madrid) and in agreement with the Spanish national legislation. Live-trapping was performed seasonally (winter: January-February; spring: June; summer: August; and autumn: October-November) during 3 consecutive years and in the main habitats of the study area: pine reforestation, deciduous forest and scrubland. The study area was divided into 9 plots (3 per habitat) separated 3 km to avoid possible replication. In each plot, we placed 25 Sherman live traps separated 10 m each and shaping a 5 × 5 grid. Traps remained on the field 9 consecutive nights (3 nights per habitat), making check-ups at dawn and dusk to minimize the time animals were captive and to avoid bias in GC levels. Traps were covered with vegetation to lessen the effect of adverse weather conditions in trapped animals and oriented against the slope to allow a correct closing. They were also partly filled with waterproof cotton wool to improve thermal insulation. We used 20 g of bread soaked in rancid oil inside traps as bait.

Captured animals were identified by external morphology. Sex and breeding condition were determined according to Gurnell and Flowerdew (2006). Thus, males presented a longer anal-genital distance. Breeding females showed prominent nipples on abdomen and thorax, and vaginal membrane perforated. In breeding adult males, testicles became bigger and marked in the scrotal sac. Individual body weight was measured employing a 100-g hand-held scale (PESNET, 100 g).

We used harmless waterproof paints (Marking stick DFV, www.divasa-farmavic.com) to identify possible recaptures. We marked the animals on paws, inner ear area and tail, where marks were less likely to degrade due to hair loss and they might remain

from one season to another. The high mortality in the study area during winter reduced the number of animals recaptured in winter and spring. Animals were more likely recaptured during summer and autumn, identifying them as re-sampled animals. Only new individuals were included in our study to avoid pseudo-replication. After handling, captured individuals were released as fast as possible in the same capture area.

Moon phase was recorded during the night before trap review, considering the percentage of night clouds. Weather conditions – i.e., rainfall, temperature and relative air humidity – were obtained from a weather station located in the study area.

Faecal samples

Traps were checked twice daily with 12 hours in between review. GC concentration peaks in faeces of related mouse species appear around 8-12 h hours after the ACTH injection (Touma et al. 2003; Touma et al. 2004; Abelson et al. 2016). Therefore, as wood mice are more active 2-4 after sunset (Montgomery and Gurnell 1985), faeces of the few individuals trapped during the afternoon were discarded to avoid bias due to any trapping effect on the physiological stress response. Thus, we only included in our study fresh faeces, i.e., not dried and with a soft texture (Navarro-Castilla et al. 2014a), from individuals trapped during the night. In this way, we made sure that the faecal samples analysed belonged to individuals trapped less than 8 hours. They were collected between sunrise and two hours thereafter to minimize exposure to environmental conditions and microbial action (Millspaugh and Washburn 2003; Möstl et al. 2005; Barja et al. 2012) and avoid the influence of circadian rhythm in excretion

patterns (Touma et al. 2003; Touma et al. 2004). Additionally, to avoid cross contamination of faecal samples, we also rejected the traps where urine was detected.

Faeces in the field were collected in Eppendorf tubes, stored in a portable cooler at 4 ◦ C, and immediately frozen at -20 ◦ C until analysed in the laboratory.

Extraction and quantification of faecal corticosterone metabolites

Frozen faecal samples were dried at 50 ◦ C in a laboratory heater (Selecta, model CONTERM 2000208) until constant weight. The extraction of GC metabolites from the faeces was performed according to Touma et al. (2003). Briefly, samples were homogenized with mortar and pestle. Then, 0.05 g were mixed with 1 ml of 80% methanol in an Eppendorf tube. Samples were shaken for 30 minutes on a multivortex and then centrifuged for 15 minutes at 2,500 × g. Supernatants were obtained diluted 1:10 with assay buffer and maintained at -20 ◦ C until analysis.

To analyse the FCM concentration in the faeces, we used an enzyme immunoassay (EIA) developed and validated specifically for wood mice in the laboratory of Endocrinology of the Veterinary Faculty (Universidad Complutense, Madrid) following the methods described in Refs. (Bamberg et al. 2001; Morrow et al. 2002; Silván et al. 2007). Polyclonal antibody (CT1098) was raised in our laboratory in rabbits against corticosterone 3-CMO: BSA (Steraloids Inc., Newport, USA). Cross reactivity of the corticosterone antibody CT1098 was: Corticosterone: 100%; Aldosterone: 10.5%; Prednisolone: 5.71%; Prednisone: 8.9%; Cortisone: 10.8%; Cortisol:

6.4%; 11-Deoxycorticosterone: 14.31%; 21-Deoxycorticosterone: 5.31%; Progesterone, estradiol, testosterone and estrone sulphate <0.1%. The low detection limit of the assay tested as defined by Abraham (1975) and Munro and Lasley (1987) and calculated from B0 values (maximum binding) minus 2SD in 10 consecutive assays was 3 ng corticosterone metabolites/g dry faeces. The addition of exogenous quantities of corticosterone (30.0; 300.0; 3,000.0 ng corticosterone/g faeces) to pooled faecal samples with high (23,568.05 ng corticosterone/g faeces) and low (534.13 ng corticosterone/g faeces) FCM concentrations showed a mean recovery of 98.96% for high FCM samples and 97.33% for low FCM samples. Intra-assay coefficient of variation (CV) was calculated by assaying ten times pools of faecal samples within an assay. Inter-assay CV was calculated by assaying the same pools of faecal samples in ten consecutive assays. Intra- and inter-assay CV were 6.5% and 10.5% in low concentrations, and 5.1% and 9.9% in high FCM concentrations. Parallelism was performed by comparing serial dilutions of pooled faecal extracts and the standard curve demonstrating that binding inhibition curves of serially diluted pools of faecal extracts were parallel to the standard curve (Line formula of standard dose response curve: $Y = 1.78 - 0.00010X$ and line formula of serial dilutions of pooled data, samples: $Y = 1.75 - 0.00010X$, $P = 0.92$, $R2 = 0.91$). FCM concentrations are expressed as ng/g of dry faeces.

To confirm suitability of the EIA for wood mouse faecal samples, a physiological validation was performed based on ACTH stimulation test, which is considered the best test to evaluate adrenal gland functionality (Behrend et al. 2013). Following the procedure described in Touma et al. (2004), we injected a high dose 60 µ g/100 g

of body weight of synthetic ACTH (Synacthen Depot, Novartis, Germany) into five captive individuals (two females and three males). Samples of each of these individuals were collected within minutes after defecation and immediately stored in Eppendorf tubes at -20 ◦ C until analysis. Sampling times were: 0, 2, 4, 6, 8, 10, 12, 14, 18, 22 and 26 hours post injection. In the biological validation experiment, measured FCM baseline levels (prior to injection) for each tested individual ranged from 13,120 to 40,420 ng/g faeces. In the five individuals, the corticosterone EIA detected an average increase in FCM concentrations ranging from 116% to 247% within 8 to 12 h of the injection event. Following, a downward trend towards baseline FCM values was detected within 12 to 18 h, validating the corticosterone EIA for the mouse faecal samples analysis.

Statistical analysis

Support Vector Machines

We employed support vector machines (SVMs) to analyse which environmental and individual factors allowed us to explain the physiological stress response of the faecal samples collected during fieldwork. SVMs are supervised statistical learning methods applicable to pattern classification and regression tasks (Vapnik 1995; Cortes and Vapnik 1995; Vapnik and Vapnik 1998; Cristianini and Shawe-Taylor 2000; Schölkopf et al. 2001; Muller et al. 2001; Friedman 2006). They have captured the attention of the scientific community as they have proven to be powerful and useful tools in a wide range of problems in different disciplines. In particular, SVM-based analyses have been successfully applied to different biological data in areas like environmental science, neuroscience, bioinformatics

and medical diagnosis between others (Guyon et al. 2002; Fagerlund 2007; Fröhlich et al. 2008; Martiskainen et al. 2009; Dosenbach et al. 2010; Luo and Cheng 2012).

SVM classifiers are machine-learning tools built to predict the class or category to which a particular object belongs as a function of an n-dimensional feature vector (χ). They are constructed adjusting by training the parameters of a classification function (Eq. 1) to get an optimal classification of a series of known feature vectors with their corresponding classes. The goal of the training phase is finding a hyperplane in the feature space separating the target classes, which provides the SVM model with the ability to generalize and predict the class of out-of-training samples. Obviously, if the training set does not contain representative samples with relevant information, no empirical model can be constructed.

A SVM classification function is as follows:

$$f(\chi) = \sum_{i=1}^{N} \alpha_i \, y_i \, K(\chi, \chi_i) + b \qquad (1)$$

where $\{(\chi_i , y_i)\}N_{i=1}$ is the training set with N feature vectors (χi) and their corresponding class (yi); αi and b are parameters to adjust during the training phase; and K (χ, χi) is the so-called kernel function. A kernel function receives two feature vectors as input and returns a single scalar value measuring the similarity between these vectors. This function is the base of the SVM learning method, since it implicitly maps original input data into a high-dimensional space where the margins separating the data are maximized (Cristianini and Shawe-Taylor 2000; Muller et al. 2001). Different suitable kernel

functions can be used in a SVM depending on the problem properties and the distribution of the samples to classify – e.g., polynomial, quadratic or sigmoid kernels (Vapnik 1995; Schölkopf et al. 2001; Muller et al. 2001). At this regard, an interesting feature of SVM-based classifiers is their ability to generate linear or nonlinear decision boundaries depending on the kernel function.

SVM analysis

Linear SVMs are fast to train and execute. However, the assumption that the data are linearly separable is rarely fulfilled. In contrast, nonlinear SVM analysis provides better performance in many problems, but it loses explanatory capacity as nonlinear kernels map the data to a high-dimensional feature space where it is difficult to interpret the relevance of each original feature. Given the nature of our analysis, to identify which factors were the main stressors of the mice trapped during fieldwork, we carried out what we called a brute force analysis. We constructed SVM models based on different kernel functions to predict the stress response according to all the possible combinations of potential stressors, i.e., 1-to-11-dimensional classifiers combining year, month, season, rainfall, temperature, relative air humidity, habitat, moon phase, sex, breeding condition and body weight. Every classifier was trained and tested on all the samples in the dataset. Then, we calculated the percentage of correct predictions in the tests. This value allowed us to identify the combinations of environmental and/or individual factors providing a better predictive accuracy and, therefore, the factors that better explained the stress response in our mice faecal samples.

Results reported in this paper correspond to SVM classifiers based on a Gaussian (Tax and Duin 1999; Schölkopf et al. 2001) and a linear kernel (Table 8). Gaussian SVMs were the classifiers with better performance regardless of the factors in the feature vector, while results of the linear SVMs are presented to compare performance of linear and nonlinear analysis.

Table 8. Definition of the Gaussian and the linear kernel function.

Kernel function	
Gaussian kernel	$K_{\sigma}^{G}(\chi,\chi_i) = e^{-\|\chi-\chi_i\|^2/2\sigma^2}$
Linear kernel	$K^{L}(\chi,\chi_i) = \chi \cdot \chi_i$

Faecal samples categorization

Experimental evidence support that physiological stress response is related to the FCM level. We thus assumed that mice could be categorized according to this value in order to study the relationship between their level of stress and different potential stressors. This categorization was made theoretically from the FCM level histogram (Fig. 24) as, to the best of our knowledge, there is no experimental evidence regarding specific threshold values determining different levels of stress as a function of the FCM concentration.

We contemplated two different scenarios. In a first group of experiments, we considered two possible classes: animals with a low and a high level of stress. Faecal samples were distributed according to the FCM level using a 50-50 splitting strategy, i.e., 50% of the samples belonged to each class (Fig. 24B). In a second group of experiments, we categorized the samples in three classes (low,

moderate and high) using a 25-50-25 splitting strategy (Fig. 24C). To address this multi-class problem, we used a one-against-all strategy (Vapnik and Vapnik 1998). We trained three different classifiers to separate each class of the other two classes, and classified out-of-training samples according to the classifier giving the largest output. Results presented in this paper correspond to the three-class distribution, but the obtained with the two-class distribution were equivalent.

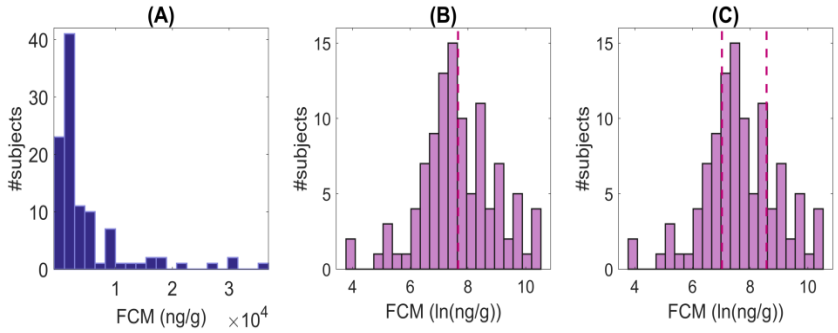


Figure 24. FCM level histogram in linear **(A)** and logarithmic scale **(B and C).** Dashed line in panel B denotes the threshold between the two categories considered in the two-class analyses. Faecal samples below this threshold are considered to correspond to animals with a low level of stress, while those above this value to animals with a high level of stress. Similarly, dash lines in panel C correspond to thresholds in the three-class analyses (low, moderate and high stress level).

Validation of the SVM classifiers

One of the major problems of SVM learning methods is incurring in overfitting (Cawley and Talbot 2010), i.e., even though all feature vectors in the training set might be well classified, the SVM might

lose its ability to generalize out of the training set. This problem for generalization was particularly tricky in an analysis like the brute-force analysis proposed here, where the training set was also used as testing set. Then, we validated our SVM models using a 10-fold cross-validation scheme (Cristianini and Shawe-Taylor 2000). Faecal samples were randomly divided into ten mutually exclusive subsets of the same size. Nine of these subsets were used as training set to build the model, while the remaining subset was used as testing set to determine the generalization ability of the SVM. We repeated ten times these steps until every subset was used as testing set. The predictive accuracy of the ten tests in the 10-fold cross-validation allowed us to validate whether the SVM model was incurring in overfitting.

In addition to the overfitting validation, the 10-fold cross-validation also allowed us to compare the predictive accuracy of different SVMs. For this, the 10-fold cross-validation was repeated and averaged 100 times for each combination of factors in the input. This average value was a metric to quantify the SVM predictive accuracy.

Data availability

The datasets analysed during the current study are available from the corresponding author on reasonable request.

RESULTS

We first study the correlation between the FCM level in the 105 fresh faecal samples collected during fieldwork and the different environmental and individual factors considered as potential stressors

in this investigation. In the case of quantitative factors, we calculate the Pearson correlation coefficient (r) with a significance level p = 0.05 (Table 9). This measure estimates the linear dependence between two variables. We also generate scatterplots characterizing these correlations (Fig. 25). Our findings point out that there is not a strong linear correlation between the FCM level and any of the quantitative factors (the r value lies between -0.31 and 0.28), being statistically significant only the results obtained for temperature and humidity ($p < 0.01$). Furthermore, as scatterplots of Fig. 25 illustrate, faecal samples cannot be linearly separated into different categories according to these factors. Nevertheless, although no general conclusions can be drawn for all the samples, it seems that the captured mice only exhibit a high stress level under some circumstances. The more stressed animals are smaller than 25 g, or are trapped when the rainfall level is below 14 l/m2 or the temperature is lower than 5 ◦ C or greater than 15 ◦ C. In the case of air humidity, samples are distributed more homogeneously.

Table 9. Correlation coefficient between the FCM level in the faecal samples and the quantitative potential environmental and individual stressors considered in our investigation

Factors	**r**	***p* value**
Rainfall	-0.11	0.27
Temperature	0.28	0.004
Humidity	-0.31	0.001

Weight	-0.05	0.64

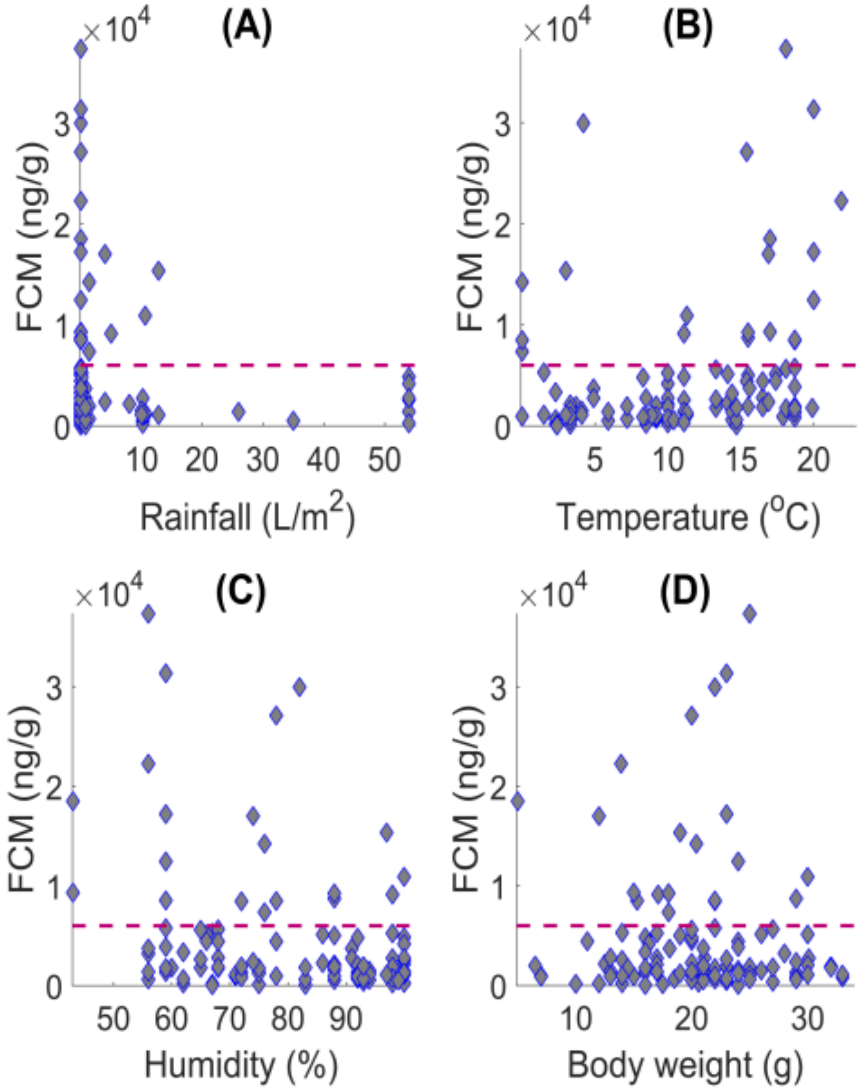


Figure 25. Distribution of the 105 faecal samples analysed in our study as a function of the four quantitative factors considered as

potential stressors. Dashed line corresponds to a FCM concentration threshold equal to 0.6 · 104 ng/g.

Results for qualitative factors are equivalent. In this case, we calculate and compare the probability distribution of the FCM concentration for each possible value (Fig. 26). In general, these are broad probability distributions that cover a wide overlapping range of FCM levels. The calculation of the corresponding means and standard deviations (Table 10) corroborates quantitatively these observations. This indicates that none single factor completely explains the FCM level in all the faecal samples. However, means and peak values in the probability distributions do allow us to identify some general trends in our data. At this regard, it is important to highlight that some of these trends can be due to a poor statistical significance. For example, the probability distributions for January and February – two winter months – differ, but we only have four faecal samples collected in January. Therefore, it is impossible to know whether the animals captured in January are mainly outliers, or whether the FCM concentration is actually higher in this month. Focusing on data with enough statistic significance, mice are generally more stressed during spring and summer than during winter and autumn. Regarding the moon phase, animals showing the highest levels of stress are captured during waxing moon. Breeding individuals tend to be more stressed than non-breeding animals, while, independently of the breeding condition, most females are more stressed than males. Finally, an interesting outcome against one of our initial hypothesis is that no significant differences appear in the stress level of animals living in different habitats.

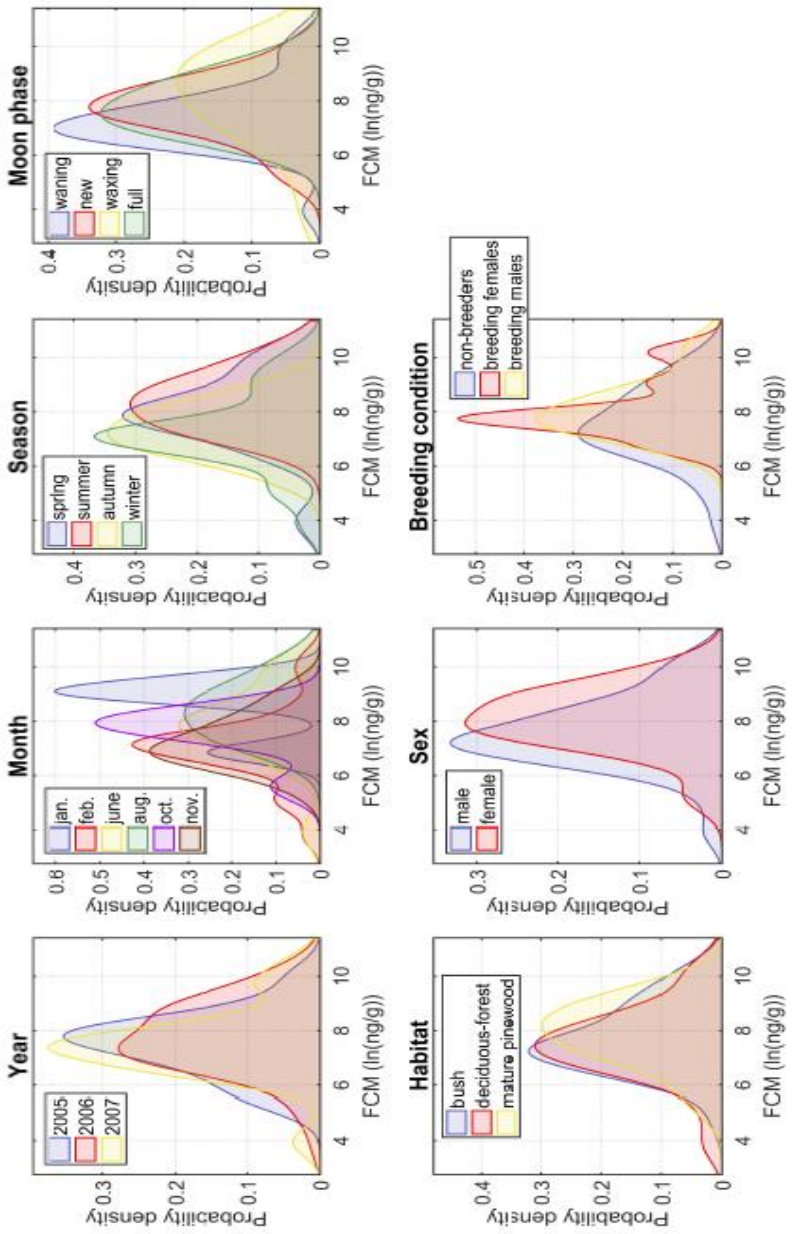


Figure 26. Comparison of the probability distribution of the FCM level according to each possible value of the seven qualitative factors considered as potential stressors in our study. See also Table 10.

Table 10. Number of faecal samples and mean FCM concentration (ng corticosterone metabolites/g dry faeces) per qualitative independent variable considered in our study.

Parameter	**# samples**	**mean ± SE**	**SD**
Year			
2005	19	3.437 ± 1.151	5.019
2006	65	5.063 ± 843	6.800
Month			
Jan. Feb.	4	7.755 ± 2.723	5.447
June	24	3.083 ± 1.327	6.499
Aug. Oct.	17	6.668 ± 2.320	9.567
Nov.	28	7.223 ± 1.514	8.010
Season			
Winter	28	3.750 ± 1.225	6.484
Spring	17	6.668 ± 2.320	9.567
Summer	28	7.223 ± 1.514	8.010
Habitat			
Mature pinewood	24	4.722 ± 955	4.680
Deciduous forest	43	4.504 ± 1.159	7.603
Bush	38	4.977 ± 1.201	7.406
Moon phase			
New	47	4.117 ± 774	5.307
Waxing	19	8.378 ± 2.203	9.602
Full	8	3.570 ± 1.217	3.443
Waning	31	3.706 ± 1.317	7.333

Sex			
Female	31	5.514 ± 1.088	6.058
Male	74	4.394 ± 843	7.250
Breeding condition			
Non-breeders	77	4.082 ± 643	5.645
Breeding females	7	6.585 ± 3.554	9.403
Breeding males	21	6.464 ± 2.123	9.731

With the analyses performed so far, we have identified the correlation among the FCM level in specific subsets of faecal samples and the value of some environmental and individual factors. However, these findings only explain the stress level of a small percentage of captured animals. It points out the complex relationships among stressors and the mice physiological stress response, where multiple factors may simultaneously influence. To elucidate these complex relationships and find out how the combination of different factors can induce a given stress level, we carried out a SVM-based analysis. Figure 27 shows the general results of our brute-force analysis. A first relevant result illustrated in panel A of this figure is the difference of using a linear and a nonlinear approach. On one hand, (nonlinear) Gaussian classifiers have a greater predictive accuracy than the equivalent linear classifier independently of the model dimension (0.84 ± 0.16 vs. 0.38 ± 0.06). On the other hand, linear classifiers have a nearly constant accuracy, while the nonlinear SVM accuracy grows and tends to one as the number of dimensions increases (cf. dark solid lines). These results are in agreement with the previous ones, pointing out that faecal samples of our dataset are not linearly separable and emphasizing the need of using a nonlinear analysis.

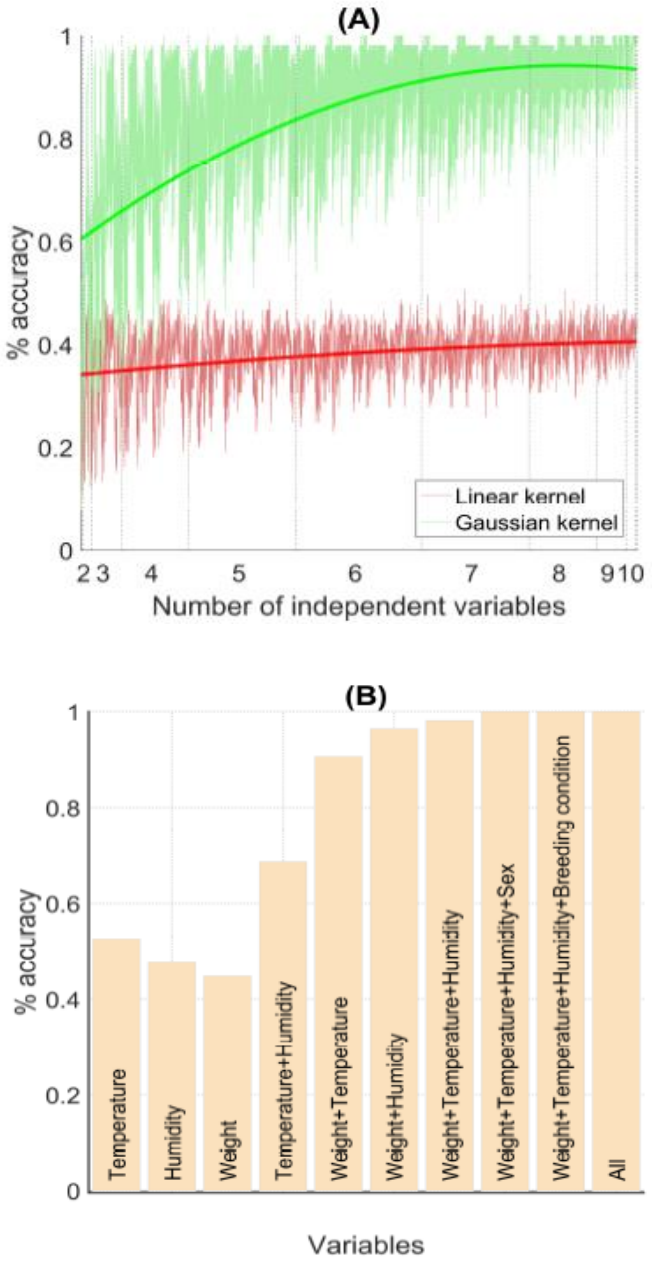


Figure 27. (A) Comparison between the predictive accuracy of Gaussian and linear SVM classifiers as a function of the number of independent variables considered to build the model. By predictive

accuracy we mean the percentage of faecal samples correctly classified. Note that in this representation we do not distinguish among the specific variables used to make the prediction, but only the number of dimensions. Dark-solid lines illustrate the global tendency. **(B)** Comparison of the predictive accuracy of some relevant Gaussian classifiers. Label inside the bar identifies the factors used to build the model. "All" corresponds to a SVM built with all the environmental and individual factors analysed in our study (11 dimensions).

Focusing on the nonlinear SVMs, some models based on only two variables are able to classify correctly more than 90% of faecal samples (Fig. 27B). In particular, the two-dimensional SVM model based on body weight and humidity and the one based on body weight and temperature predict properly 101 and 94 out of 105 samples, respectively. Predictive accuracy for the rest of two-dimensional models is below 0.75. These results suggest a strong correlation between the mouse's weight and the FCM level. However, it is important to keep in mind that the corresponding correlation coefficient (Table 9) indicates a weak correlation between them ($r = -0.05$). Similarly, predictive accuracy of the one-dimensional classifier built only considering the body weight is 0.46. These observations make us argue that the FCM level is non-linearly correlated with the combination of body weight and humidity and/or temperature. Note that these are the variables with a stronger linear correlation with the FCM concentration (Table 9). In line with this result, the one-dimensional SVMs based on humidity and temperature are the ones providing the best predictive accuracy (0.64 and 0.60, respectively).

Results obtained with more-than-two-dimension classifiers agree with these findings. Multiple combinations of three factors allow us to predict the level of stress of more than 90% of samples in the dataset. The three-dimensional model with the greatest predictive accuracy is the one based on body weight, temperature and humidity (103 out of 105 samples). In the rest of cases with an accuracy greater than 90% (16 possible combinations), the model is based on body weight and all the possible combinations of temperature or humidity with the rest of factors. Predictive accuracy for four-dimensional models is always close to 100%. In particular, two combinations of four factors (weight, humidity, temperature and sex/breeding condition) allow us to classify correctly all the samples in the dataset. Here on, predictive accuracy is 100% for different combinations of variables, but in all cases body weight, humidity and temperature participate in the model.

Finally, Figure 28 compares the results of the brute-force analysis and the 10-fold cross-validation. As expected, predictive accuracy in the cross-validation is lower, since the testing faecal samples now are not included in the training set. However, the general trends described above are kept. Body weight, air humidity and temperature are the factors that provide better predictions. Furthermore, in the 10-fold cross-validation the three-dimensional SVM classifier based on these factors shows the best mean predictive accuracy (0.84 ± 0.01). In general, the difference between accuracy in the brute-force analysis and the cross-validation is low, which allows us to be confident that our models are not incurring in overfitting and, therefore, validates the generality of our results. Then, we can conclude that body weight, air humidity and temperature are the

factors that better explain the FCM concentration in the collected mice faecal samples.

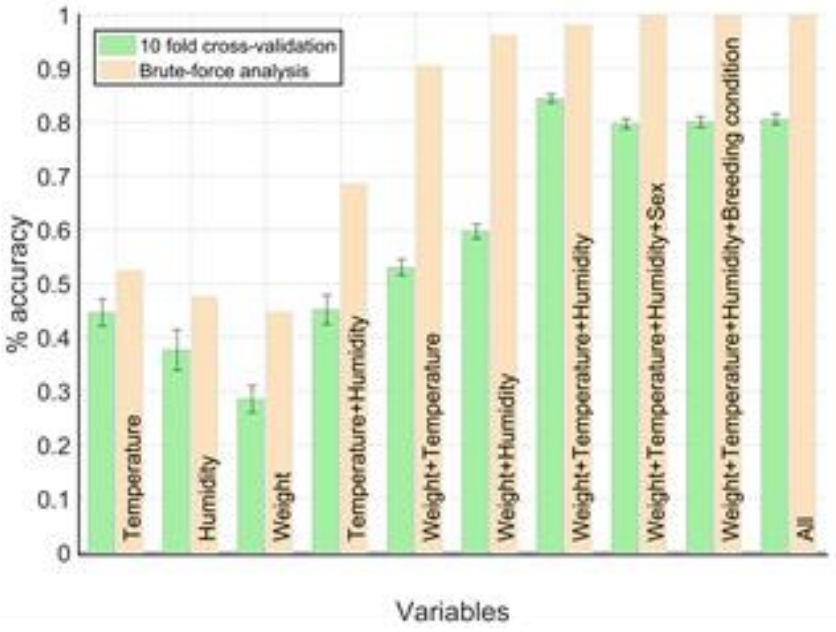


Figure 28. Results of the 10-fold cross-validation and comparison with the results obtained in the brute-force analysis (cf. Fig. 27B). Average data are generated repeating the 10-fold cross-validations with different random seeds.

DISCUSSION

Physiological stress response is defined as an adaptive response executed by organisms in order to cope with unpredictable situations (Möstl and Palme 2002). Both environmental and individual factors can act as stressors in wild mammal populations, triggering the physiological stress response (Reeder and Kramer 2005; Barja 2015). In this investigation, we study the effect of different potential stressors

in 105 wild wood mice captured in Montes do Invernadeiro Natural Park (Galicia, Spain). We characterize the physiological stress response by means of the FCM concentration, which reflects free GC in plasma and yields an accurate profile of the adrenocortical activity (Wingfield et al. 1997; Goymann et al. 1999; Touma and Palme 2005). We have identified different relationships among some environmental and individual factors and the level of stress observed in a given animal. Some of these relationships are expected. For instance, breeding individuals and females in average show a higher level of stress than non-breeders and males, respectively (Fig. 26). These observations are in agreement with previous studies on other rodent species and habitats (Touma et al. 2004; Dantzer et al. 2010; Navarro-Castilla et al. 2014b; Navarro-Castilla and Barja 2014b) . The request for maternal investment during pregnancy and lactation could explain the higher FCM levels in breeding females. Furthermore, breeding females often experience multiple metabolic changes where GC play a major role (Tataranni et al. 1996; Bauman 2000; Strier et al. 2003; Reeder and Kramer 2005). In males, the competition for mating females increases aggressive behaviour what might explain the increased FCM concentration in breeding males. The different mean FCM level in females and males agrees with the observation of a different basal GC level between sexes due to differences in the metabolism and excretion rate (Handa et al. 1994; Touma et al. 2003). We also observe that stress is commonly greater in animals captured in June and August (Fig. 26). This result could be also related to physiological and/or behavioural changes induced by breeding, since the reproductive period in the study area occurs in these months (late spring and summer) (Torre et al. 2002). In other cases, we detect that a

specific stress level is correlated with the value of a given stressor (Fig. 25). For instance, the highest levels of stress occur for animals captured during a specific moon phase, when the rainfall level is below a given threshold, or for too low or too high temperatures. All these findings allow us to understand the response of an average individual to a stressor or even to specific combinations of stressors. Nevertheless, they do not explain the variation of the FCM concentration in all the faecal samples of our dataset because of the broadness and overlapping of the FCM level distributions for all the factors analysed. This result corroborates that physiological stress response is a complex physiological response where multiple factors interact simultaneously in a nonlinear manner.

To address the study of the underlying mechanisms triggering a specific stress response, we use a SVM-based approach. SVMs present two relevant properties for our study. On one hand, SVM classifiers have the ability to learn complicated decision boundaries when the data present complex distributions. On the other hand, we have a small dataset with a relative large dimensionality of the feature space. This represents an obstacle for many statistical methods, because, in general, the larger the set of available samples, the better the generalization ability (Raudys and Jain 1991). However, SVMs estimate the separating hyperplanes by means of a limited number of training samples characterizing each class (the support vectors). It permits obtaining an optimal classification performance in high-dimensional problems and/or with a low ratio of training samples versus dimensions of the input data (e.g., see Refs Belousov et al. 2002; Steinwart 2004; Guo and Dyer 2005).

The constructed SVMs verify that multiple factors may directly or indirectly influence the level of stress in a wild wood mouse, but the ones that better explain the stress response in the 105 captured animals are body weight, relative air humidity and temperature. Figure 29 illustrates the complex interactions among these three factors to produce a specific level of stress in an individual. Classifiers built with only an individual (body weight) and an environmental factor (relative humidity or temperature) are able to explain the increase in the FCM levels observed in more than 90% of faecal samples. This increase can be completely explained by the combination of the three factors. The corresponding three-dimensional model is able to predict the stress response in more than 98% of captured mice. Thus, many of the correlations found in the average FCM levels do not seem to be so significant as one could initially expect. In particular, we would like to highlight the role of seasonality and the dependence of the basal FCM level on sex and breeding condition. Predictive accuracy of linear and Gaussian three-dimensional classifiers based on these variables are 0.48 and 0.56, respectively (cf. accuracy for models based on body weight, air humidity and temperature). This does not mean that these factors do not influence the stress response. As we discuss above, it is obvious that they do. However, under some circumstances, their effect can be masked by stressors with a stronger impact. For instance, multiple climatic factors can fluctuate within a season, which may bias the dependence of the FCM level on seasonality. Indeed, several investigations on *A.* sylvaticus have reported an increasing food availability during unfavorable climatic seasons (Montgomery 1989; Montgomery et al. 1991; Díaz et al. 2010). In other cases, a variable

or combination of variables summarizes the role or effect of other stressors. For instance, the trade-off between temperature and humidity better characterize favorable and unfavorable climatic periods than seasonality; and, at the same time, specific combinations of temperature and humidity are correlated with the reproductive period or with a given habitat.

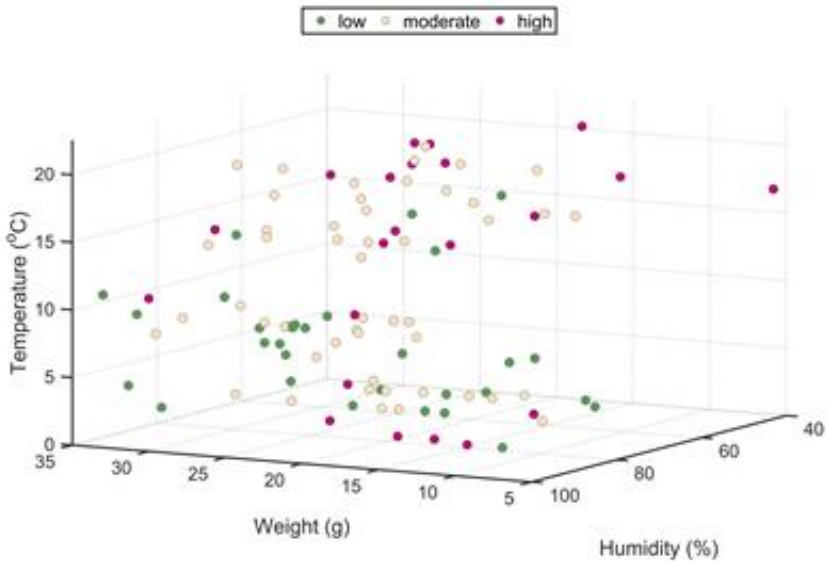


Figure 29. Distribution of faecal samples in our dataset in a three-dimensional space formed by body weight, temperature and relative air humidity. Each colour corresponds to a category (low, moderate and high FCM level).

Our results seem to be partially related to the role played by temperature and humidity in primary production. Previous studies have attested the important relationship between climatic conditions and the state of small mammal populations (Lima and Jaksic 1999; Meserve et al. 2001; Díaz et al. 2010). In this sense, temperature and

humidity have a significant influence on trophic resource availability and vegetation cover thickness (Shaver et al. 2000; Ciais et al. 2005; Slaney et al. 2007). During favorable climatic periods, a large number of seeds, fruits and invertebrates are available for small mammal species like *A. sylvaticus*. This food availability produces a body condition improvement in rodents (Montgomery et al. 1991; Rosário and Mathias 2004; Sunyer et al. 2016). Vegetation cover also provides shelter against predators (Perea et al. 2011; Rosalino et al. 2011a). In this manner, an optimal trade-off between temperature and relative humidity can modify the stress level in a wood mouse in two different ways. Firstly, by increasing the food availability, and hence improving the individuals' body condition. Secondly, by causing an appropriate growth of the vegetation cover, which involves an improvement in predator avoidance. An interesting result related to weather conditions is the weak correlation between the FCM concentration and the rainfall level as compared with other climatic factors. As temperature and humidity, rainfall significantly influences primary production. During rainy periods, food availability usually grows and the vegetation cover is denser (Rosenzweig 1995; Mittelbach et al. 2001; Brown and Ernest 2002). Due to this, rainfall is often considered one of the main environmental stressors for small mammals. Nevertheless, our SVMs does not identify it as a critical factor triggering the stress response. A possible explanation to this result is the weather conditions in the study area. Ambient humidity in Montes do Invernadeiro Natural Park is in general high during the whole year and it is not directly associated to the fallen rain (Fig. 30A). With this high ambient humidity levels, this has a similar effect to rainfall on trophic resources and vegetation cover, even during dry periods. This makes

humidity a more informative variable. This result could suppose a threat for the generalization of our findings. However, we argue that the situation observed in Montes do Invernadeiro Natural Park is a common situation in multiple habitats. Then, we hypothesize that, in general, humidity could be a stronger stressor in small mammal than rainfall, both if humidity directly correlates with rainfall and if it has a distinct origin (a nearby water stream, night dew, fog, etc).

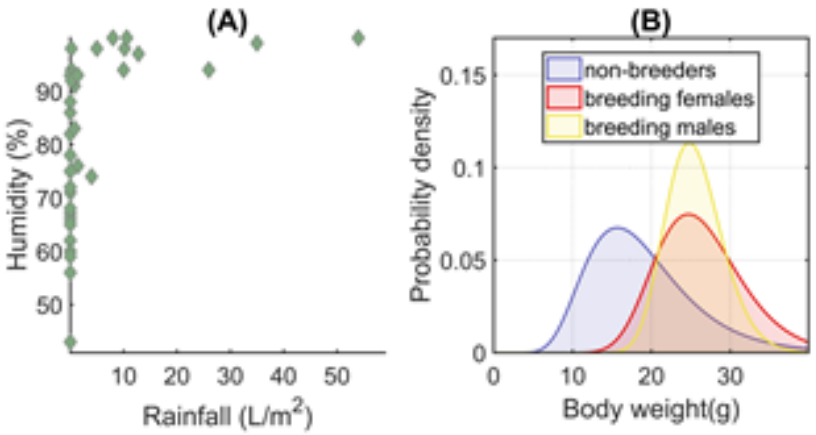


Figure 30. (A) Correlation between rainfall and relative air humidity. Note that in the study area air humidity is not always related to rainfall. The correlation coefficient between these two variables is r = 0.46 with a significance level $p < 0.001$. **(B)** Probability distributions of the body weight for each possible breeding condition.

Although at a first glance body weight does not appear to be strongly correlated with the FCM level (see Fig. 25 and Table 9), our SVM models point out that it is a highly informative variable. Our interpretation of this result is that the animal's weight is linked to multiple causes of stress producing different responses depending on

the context. Weight is an indirect measure of body size and condition, so it is closely associated with the ability of individuals to face up the wide range of daily threats – e.g., it is supposed that stronger individuals may more successfully avoid predation than weaker ones (Montgomery and Gurnell 1985; Wauters and Dhondt 1989a). Body weight has also a strong correlation with the mice's age and, therefore, with their breeding condition (Fig. 30B). Its correlation with the age also makes weight a suitable indicator of the social range and behaviour of an animal in the population. According to this, it has been observed that subordinate individuals (generally juveniles and sub-adults; 0-20 g (Lewis 1968b) tend to occupy poor quality habitats, with lower food availability and higher predation risk due to territorial behaviour and intraspecific competitions (Wauters and Dhondt 1989b; Bondrup-Nielsen and Ims 1990). Territorial exclusion also affects to the home range size, and therefore to the distance animals have to travel in order to fulfill their energetic demands (Dickman et al. 1991). Thus, it seems plausible that younger and smaller individuals exhibit higher concentration of FCM as their access to resources is restricted and they occupy areas with less dense vegetation cover, which implies poorer body condition and an elevated predation pressure. Our outcomes show that this negative effect is more evident with extreme climatic conditions – cold temperatures and low relative humidity in our case (cf. red points for less-than-20-gram individuals in Fig. 29). At the same time, heavier individuals are commonly breeders. This makes it reasonable to suppose an increased FCM level in these animals during the reproductive period. Thus, many of the captured mice whose weight is greater than 20 g exhibit a high stress level with warm temperatures and an intermediate/low relative humidity. These

climatic conditions can be linked up with the reproductive period in the study area (late spring and summer). In contrast, during the rest of the year, these animals show lower FCM levels (cf. the concentration of green points in Fig. 29 for more-than-20-gram animals when temperature is below 15 ◦ C). These results emphasize again the complex interaction among stressors to produce a context-dependent physiological stress response.

In brief, our study points out that traditional statistical methods may not be enough to fully understand the causality among stressors and the stress response in wild mammals. This could be a common situation in multiple complex biological process, where we often analyse small datasets, with complex distributions and in a large number of interacting dimensions. Our results demonstrate that the usage of SVMs is a reliable statistical approach in this scenario. The SVM models built in this work allow us to identify meaningful correlations not found with more traditional analysis. In particular, the animal's weight and climatic factors such as relative air humidity and temperature appear to be accurate factors to explain and predict the variation of the FCM level in the captured wood mice. Our findings suggest a close correlation between weight and body condition, which seems to have a direct effect on intraspecific relationships, e.g., social dominance and territorial behaviour, and the stress response they produce. Temperature and humidity are factors to take into account due to their significant influence in vegetation, being optimal climatic conditions crucial for the survival of small mammals. In contrast, factors traditionally considered the main stressors in small mammals, e.g., sex, breeding condition or seasonality, seem to have a secondary role in the stress response of our wood mice. One threat to validity of

our findings is the small number of samples, mainly in the case of some subgroups, e.g., breeding females. One of the expected benefits of using SVMs is their potential generalization ability with a limited number of representative samples (i.e., training samples containing relevant characteristic information). This seems to be the case of our breeding females, since they present patterns previously reported in literature (e.g., higher mean levels of stress than males and non-breeding females). Unlike breeding females, other subgroups including a small number of samples, e.g., the four animals captured in January, do not appear to contain representative samples. However, our results show that the stress response is not highly connected with the corresponding factors. For instance, specific combinations of weather conditions are more informative than month or season to identify favorable and adverse periods linked up with low or high stress responses. In addition, the 10-fold cross-validation empirically corroborates the theoretical ability to generalize of our SVM classifiers (Fig. 28). Therefore, taking into account the above, we conclude that our findings are valid and general for the study area. Regarding the generalization of the findings beyond our study area, it is important to emphasize that, due to the characteristics of this region, we did not incorporate to our models some highly relevant potential stressors, e.g., different forms of human disturbance. It is obvious that these factors can play a key role in the small mammals' stress response depending on the habitat. However, we consider that our main findings are general enough to argue that they would also apply to habitats with a limited human presence, and also to other wood-mouse-like small mammal species. The only difference could be that in habitats with certain climatic conditions the role of rainfall might

change and substitute humidity as a key climatic factor. We hypothesize that in habitats with a greater human presence, human disturbances would be additional context-dependent environmental factors interacting with body weight and the key climatic factors. To validate these hypotheses and fully understand the physiology and behaviour of small mammal populations, further long-term studies including detailed climatic data of varied climatic regions should be performed.

CONCLUSIONES GENERALES

1. El ratón de campo respondió comportamentalmente ante los diferentes grados de riesgo de depredación por su depredador principal en el área de estudio, el zorro rojo.

2. En general, los machos fueron los individuos que más evitaron las trampas con olor fecal de depredador, debido a sus menores requerimientos energéticos con respecto a las hembras. Sin embargo, esta situación cambió cuando los machos estaban reproductivamente activos. Esto parece deberse a que su menor implicación en la cría de la descendencia les permite correr más riesgos, además de que durante la época reproductora los machos aumentan sus áreas de campeo hasta cinco veces más de lo habitual, lo que aumenta la probabilidad de ser capturados.

3. Los individuos reproductores evitaron las trampas de máxima concentración de olor fecal de depredador, ya que a pesar de sus altos requerimientos energéticos parecen evitar las situaciones de riesgo extremadamente elevado para asegurar la supervivencia propia, y por tanto, de las crías.

4. El ratón de campo activó la respuesta fisiológica de estrés, aumentando dicha respuesta a medida que se incrementaba la concentración de olor fecal de depredador. Así, los niveles de metabolitos de corticosterona fecal (MCFs) durante la fase experimental aumentaron significativamente en la parcela de riesgo bajo y muy significativamente en las parcelas de riesgo medio y alto, lo que parece indicar una capacidad por parte del

ratón de campo para estimar el grado de riesgo al que se enfrenta y adaptar su respuesta.

5. Los individuos reproductores (machos y hembras) mostraron mayores niveles de MCFs durante la fase experimental. Esto parece deberse a los elevados niveles de glucocorticoides observados durante la gestación y lactancia en el caso de las hembras, y a la dura competencia entre machos que ocurre durante el periodo reproductor para proteger la cópula. Durante la fase de post-tratamiento los niveles de GCs en individuos reproductores continuaron elevados aunque con menor intensidad, lo que parece indicar que la degradación de los compuestos volátiles de las heces del depredador es un factor a tener en cuenta a la hora de estimar el riesgo.

6. El ratón es capaz de discriminar los diferentes grados de riesgo a los que se enfrenta, aumentando la ingesta cuando el riesgo de depredación es máximo (100% olor de depredador). Según la hipótesis de asignación de riesgos, la situación de alto riesgo es detectada previamente y durante su captura en la trampa, haciendo de esa trampa un refugio donde poder aumentar su ingesta para hacer frente a esa situación de riesgo elevado.

7. La degradación durante cinco días de los compuestos volátiles fecales no parece ser suficiente para inactivar la respuesta comportamental, aunque la menor ingesta observada sugiere que es un factor a tener en cuenta a la hora de la toma de decisiones por el ratón de campo.

8. La variación en la ingesta de alimento mencionada previamente estuvo modulada por los factores individuales sexo y estado reproductor. Así, las hembras y los individuos no reproductores fueron los que más alimento consumieron. Esto puede explicarse por las superiores demandas energéticas de las hembras en relación con los machos, y porque los individuos reproductores prefirieron no asumir riesgos para asegurarse un buen cuidado de la descendencia.

9. La experiencia adquirida también fue un elemento importante a la hora de tomar una decisión sobre la ingesta. De este modo, los individuos recapturados redujeron la ingesta ante la presencia de un depredador ya conocido. Estos resultados sugieren que tras una experiencia positiva (el ratón no sufrió daño y fue liberado tras la manipulación), el segundo encuentro con una marca olorosa del mismo depredador es percibido como una amenaza menor, y por lo tanto la respuesta comportamental activada es de menor intensidad.

10. Los niveles medios de metabolitos de testosterona fecal también influyeron en la ingesta de alimento del ratón de campo. Así, los individuos con menores valores de testosterona fecal, las hembras y los individuos no reproductores fueron los que más alimento consumieron. Esto parece deberse a los mayores requerimientos energéticos que tienen las hembras y a la mayor disponibilidad de alimento que tienen los subordinados, generalmente individuos no reproductores, en el interior de las trampas.

11. Además, el peso de los individuos, la humedad y/o la temperatura parecen explicar más del 98% de la variación observada en la respuesta fisiológica de estrés del ratón de campo.

12. Los individuos con una mejor condición corporal (más peso) mostraron unos niveles de MCFs menores debido a su mayor capacidad para hacer frente a los estresores. Esto podría explicarse por la relación existente entre peso y rango social. Generalmente lo individuos dominantes ocupan los hábitats de mejor calidad (más protección y alimento), mientras que los subordinados (juveniles y subadultos de menor peso) son excluidos a hábitats de peor calidad lo que les hace estar peor preparados para responder ante los estresores.

13. La humedad y la temperatura explicaron también el estado fisiológico de estrés del ratón de campo. Estas variables parecen estar directamente relacionadas con la calidad del hábitat. Así, la elevada humedad y las temperaturas moderadas favorecerían el desarrollo de una abundante cobertura vegetal, y por consiguiente un aumento de la cantidad de alimento disponible y de la protección frente a los depredadores.

14. Por otra parte, las hembras y los individuos reproductores fueron los que mayores niveles de MCFs mostraron, aunque ni el sexo ni el estado reproductor parecen explicar directamente la variación en los niveles de estrés del ratón de campo.

15. Finalmente, Support Vector Machines ha demostrado ser una herramienta eficaz para predecir con exactitud las variables que intervienen en la respuesta fisiológica de estrés del ratón de

campo, siendo un método novedoso y con un amplio campo de aplicación en estudios futuros.

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ANEXO

Los capítulos de esta tesis doctoral han sido publicados o están en revisión en las siguientes revistas incluidas en el *Science Ctation Index:*

Chemoecology (2017) 27:115–122
DOI 10.1007/s00049-017-0237-1

CHEMOECOLOGY

ORIGINAL ARTICLE

Wood mice modify food intake under different degrees of predation risk: influence of acquired experience and degradation of predator's faecal volatile compounds

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Received: 25 April 2016 / Accepted: 9 February 2017 / Published online: 2 May 2017

Abstract Behavioural changes in response to predation risk could increase prey survival, but there are associated biological costs. We assessed whether wood mice (*Apodemus sylvaticus*) were able to modulate food intake based on different degrees of predation risk by one of its main predators in the study area, the red fox (*Vulpes vulpes*). Furthermore, we also examined the influence of acquired experience and degradation of the predator's faecal volatile compounds. Wood mice response was analysed by live trapping in four plots during a control phase where all plots were not treated, an experimental phase in which plots were subjected to different concentrations of fresh red fox faeces, and, finally, a post-treatment phase without renewing the faeces. All traps were provided with 4 g of roasted corn, and food intake was calculated based on the amount of bait that remained in each trap. Food intake significantly increased during the experimental phase, especially in the plot treated with the highest concentration of red fox faeces, and also throughout the post-treatment phase. Females and non-breeding individuals showed higher food intake. In addition, there was less food intake in recaptured than new individuals. Our results indicated that wood mice are able to detect different degrees of predation risk and respond accordingly by changing feeding behavioural response; the conflict between food intake and antipredatory behaviour seems to vary with faecal mark strength, which seems to lesson over time. Finally, experience acquired could also play an important role in their daily decision-making process.

Keywords Behavioural changes · Biological costs · Degrees of predation risk · Experience · Food intake · Volatile compounds

Introduction

For prey species, survival in the wild is largely affected by the ability to detect auditory, visual, and chemosensory cues of their predators (Lima and Dill 1990; Kats and Dill 1998; Becker and Gabor 2012; Hettyey et al. 2012; Navarro-Castilla and Barja 2014a). Facing a wide range of existing cues, nocturnal and crepuscular animals, especially mammals, uses scent marking as a primary means of exchanging both intra- and interspecific information (Gorman 1990; Torre et al. 2002; Wyatt 2003; Monclús and de Miguel 2003; Brennan and Kendrick 2006). Consequently, territorial marking carried out by most carnivores, which deposit different glandular secretions (Albone and Perry 1976; Asa et al. 1985), urine (Jorgenson et al. 1978), and faeces in conspicuous substrates, becomes a vital information system for nocturnal prey species (Jones and Dayan 2000; Monclús et al. 2005; Fendt 2006; Rouco et al. 2011). A sudden encounter with a predator is usually fatal for prey; consequently, chemical recognition could be a vital advantage, because, in a nocturnal setting, smell is one of the most important senses for anticipating a possible fatal encounter (Apfelbach et al. 2005).

Currently, it is well known that predation strongly influences many aspects of small mammal ecology, both directly by altering population densities and indirectly modifying their daily activities, such as reducing foraging,

Handling Editor: Thomas Schmitt.

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sociability, and grooming, interrupting reproductive activity, or restricting habitat use (Apfelbach et al. 2005; Díaz et al. 2005; Preisser et al. 2005; Creel et al. 2007; Navarro-Castilla and Barja 2014a, b). These antipredator adaptations often enhance fitness, and also reflect an apparent trade-off between costs (e.g., higher energetic expenditure or lower mating success) and benefits (e.g., not being injured) that are present during predator–prey interaction (Preisser et al. 2005). Understanding the costs and benefits of avoiding predators' cues is a major challenge in field of behaviour. Many experimental studies have analysed the variability in an animal's decision-making under both indirect (e.g., shelter or moon light) and direct (e.g., predator cues) predation risk (Kotler et al. 1991; Daly et al. 1992; Perea et al. 2011; Navarro-Castilla and Barja 2014b; Busch and Burroni 2015). However, to our knowledge, no study has examined mammalian prey ability to detect and respond to different concentrations of chemical cues (degrees of predation risk) in the wild. However, positive results obtained in aquatic environments (Kesavaraju et al. 2007) showed the importance of this approach. Furthermore, except for the work conducted by Hegab et al. (2014) under laboratory conditions, little is known about the possible variation in antipredatory response due to the degradation of volatile compounds from predator faeces. This degradation process occurs naturally in the wild, and prey could also consider it as an informative indicator of distance from or proximity to predators.

Behavioural response of small mammals seems to be triggered by a generalised response to volatile sulfurous metabolites present in faeces (Dickman and Doncaster 1984; Dickman 1992) and urine (Nolte et al. 1994) of carnivorous species that are derived from the digestion of animal proteins (Woolhouse and Morgan 1995). However, based on the existence of general compounds from a carnivorous diet, recent studies revealed a close relationship between behavioural response and coevolution of the involved species, which highlighted specific prey–predator recognition (Griffin et al. 2000; Blumstein et al. 2002; Fendt 2006; McEvoy et al. 2008; Navarro-Castilla and Barja 2014a; Busch and Burroni 2015). Consequently, experience acquired by an individual wood mouse could help inform the prey's decision-making.

Consequently, the aim of the present study was to first analyse the effect of different degrees of predation risk from a natural predator (red fox) on food intake by wood mice, particularly based on breeding condition and sex of individuals. Wood mice are essential prey in the diet of most nocturnal carnivores, such as red foxes, and their ability to recognize and avoid fox faecal odour is well known (Dickman and Doncaster 1984; Navarro-Castilla and Barja 2014a). According to the predation risk allocation hypothesis (Lima and Bednekoff 1999), individuals should increase feeding effort under low-risk situations, and should decrease feeding effort under high predation risk. However, if animals are exposed to long periods of high risk, they will be forced to resume feeding to meet their energy requirements. Therefore, we hypothesized that wood mice would change their feeding behavioural response depending on the perception of predation risk. Thus, we expected that mice would decrease food intake in response to an increase in the degree of predation risk previously perceived before going into the trap and while in the trap (likely a safer place), because red fox faecal odour is expected to also be detected inside the trap. Furthermore, we examined the effect of predator faecal volatile compound degradation and the experience acquired by wood mice on food intake, and predicted that food intake would increase in both cases because of the reduction of perceived predation risk.

Materials and methods

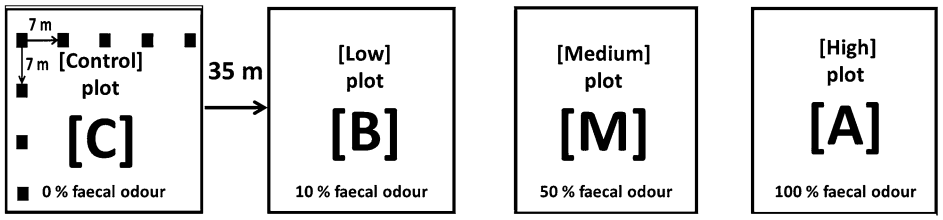
Study area

Fieldwork was conducted in a holm oak forest in Valdelatas (Madrid, Spain) at an altitude of 650 m a.s.l. within the Mediterranean climatic region. The study area is mainly composed of dense forests of holm oak (*Quercus ilex ballota*) and pine reforestations (*Pinus pinea* and *Pinus pinaster*). Scrubland vegetation includes gum rock roses (*Cistus ladanifer*), umbel-flowered sun roses (*Halimium umbellatum*), and thyme (*Thymus zygis*) as the most representative species.

Live trapping and data collection

Field experiments were conducted between February and March 2014. The response of wood mice to different concentrations of predator faecal odour was studied by live trapping. In the study area, we selected four plots separated by 35 m, and placed 20 Sherman® live traps in each plot in a 4 × 5 grid with 7 m of separation between traps (Fig. 1). All traps were oriented against the slope to avoid interference with closing and problems due to adverse weather conditions.

Live trapping was conducted in three phases with different goals: control, experimental, and post-treatment. First, in the control phase, no plots had faecal odour, so we could infer the baseline food intake levels. Next, there was an experimental phase, in which one plot acted as a control and each of the other three plots was subjected to one of the three different concentrations of faecal odour, to evaluate the feeding response of wood mice to different degrees of



Fresh faeces of red fox (g)	0	100	500	1 000
Soil of Valdelatas (g)	1 000	900	500	0
Distilled water (ml)	250	250	250	250

Fig. 1 Experimental plots and their associated treatments: *C* (control, without odour), *B* (10%, low concentration), *M* (50%, medium concentration), and *A* (100%, high concentration). Distance between traps within each grid and separation between plots is also shown

red fox predation risk. In this phase, 10 g of faecal material was placed outside of the trap on one side of the trap entrance to avoid blocking the entry for rodents but close enough to act as a potential risk for each individual trap. The faecal odour was renewed daily at sunset to ensure correct odour effectiveness when mice are more active (i.e., 2 or 4 h after dusk) (Wolton 1983; Montgomery and Gurnell 1985). Finally, in the post-treatment phase (without faecal odour renewal), we evaluated the effect of red fox faecal volatile compound degradation on food intake. All phases lasted five consecutive nights. In the post-treatment phase, we only analysed the first 2 days for which data were collected because of the fewer number of subsequently captured individuals.

Trapping sessions were conducted during days close to a new moon phase, when wood mice are more active because of reduced predation risk (Kaufman and Kaufman 1982; Díaz 1992; Navarro-Castilla and Barja 2014b), and traps were checked twice daily, at dawn and dusk, to minimize the time that animals were kept. Total trapping effort was 2400 trap nights (20 traps per grid × 4 plots × 15 nights × 2 trapping sessions).

Each captured individual was identified to species by analysing external morphology, and checked for sex and breeding condition following the protocols described by Gurnell and Flowerdew (2006). Sex was determined using anal-genital distance, which is shorter in females than males. In breeding adult females, the nipples on the abdomen and thorax are noticeable and the vaginal membrane appears perforated; in breeding adult males, the testicles enlarge quite markedly and usually descend into the scrotal sac. Body weight was measured using a 100-g hand-held scale (PESNET, 100 g), and age class was estimated based on body weight (Lewis 1968; Behnke et al. 1999): animals that weighed between 0–13 g were considered juveniles, >13–20 g were considered subadults, and >30 g were considered adults. All captured animals were marked in non-conspicuous areas with harmless waterproof paints (Marking stick DFV, www.divasa-farmavic.com) to identify possible recaptures and avoid pseudoreplication. All captured animals during each check were released quickly at the same place of capture.

Simulation of different degrees of predation risk by faecal odour

Because wood mice have previously been documented to avoid red fox faeces within an area (Navarro-Castilla and Barja 2014a), the predator treatments were made with fresh red fox faeces collected from a pair of captive animals (one male and one female) reared in the Centro de Naturaleza Opennature Cañada Real (El Escorial, Madrid). We only used fresh faeces (i.e., those which had a layer of mucus, a high level of hydration, and strong odour) (Liu et al. 2006). All faeces were collected at dawn and were frozen at −20 °C until used in the experiments to prevent degradation of volatile compounds (Martín et al. 2010). In carnivores, volatile compounds vary in relation to seasonal or individual factors such as sex, age, and breeding condition (Raymer et al. 1984; Andreolini et al. 1987; Hayes et al. 2006). For this reason, all collected red fox faeces were thawed for 1.5 h, and then mixed to obtain a homogeneous mixture. Thus, we ensured a similar initial degree of predation risk to prevent possible bias in our results. Three different predator treatments were established that differed in red fox faecal concentration, and each was applied to a plot: low (10%), medium (50%), and high (100%) concentrations. There was also an odourless control (0%) (see details in Fig. 1). Then, 10 g of each faecal treatment was frozen at −20 °C until the start of field experiments.

Food intake assessment

All traps were baited with 4 g of toasted corn. The remains of unconsumed bait by each captured individual were

collected and dried at 80 °C in a heater for 1 h, and then weighed with an electronic balance (C-3000/0.01 g CS, COBOS; precision 0.01 g) to determine the amount of food eaten in each trap by each individual.

Statistical analysis

Food intake under predation risk was analysed by general linear model (GLM) using the amount of food taken (g) corrected by the weight of the animals (g) as the response variable to avoid a possible bias in our results due to differences in consumption based on an individual's weight. The response variable was transformed ($\log_{10} + 1$) to produce a normal distribution (Shapiro test) and homogenous variance (Levene test). The independent variables were phase (control/experimental/post-treatment), sex (male/female), breeding condition (breeding/non-breeding), and recapture (new/recaptured) as fixed factors. Furthermore, because of their biological importance, the interactions between phase and plot, and between phase and recapture were also included in the GLM. Significant factors that influence food intake were analysed by Tukey's honestly significant difference (HSD) post hoc tests. In addition, because of the significant interaction of phase × plot, ANOVA tests were performed for each plot to analyse individual differences in food intake during the three phases. Finally, we analysed the influence of experience acquired regarding food intake during each phase by performing *T* tests between new individuals and recaptured ones. Results were considered significant at $\alpha < 0.05$. Data are represented as mean ± standard error (SE). Statistical data analysis was performed in SPSS 22.0 for Windows (SPSS Inc, Chicago, IL, USA).

Ethics statement

This research complies with the regulations on the protection of animals used for scientific purposes (Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 and the Spanish legislation (Royal Decree 53/2013). The study had the approval of the Autonomous Community of Madrid (reference number 10/211643.9/13) and favourable reports from both the Ethics Committee of the Autonomous University of Madrid and the Body Enabled (CIS 50-940-A007).

Results

The total number of wood mice captured was 253 individuals, of which 169 were new and 84 were recaptured. The GLM showed that the significant factors that explained the variation in food intake were phase ($F_{2,173} = 8.044$, $p < 0.0001$), sex ($F_{1,173} = 9.665$, $p = 0.002$), breeding condition ($F_{1,173} = 17.614$, $p < 0.0001$), and interactions between phase and plot ($F_{9,173} = 2.107$, $p = 0.030$) and between phase and recapture ($F_{2,173} = 3.052$, $p = 0.049$) (see more details in Table 1). Wood mice significantly increased food intake during the experimental (Tukey's HSD, $p = 0.004$) and post-treatment phases (Tukey's HSD, $p = 0.001$) compared with the control phase. However, differences were not significant between the experimental and post-treatment phases (Tukey's HSD, $p = 0.505$) (Fig. 2). The significant interaction between phase × plot showed that food intake varied between plots during the three phases (Fig. 3). Individual ANOVA tests performed for each plot revealed that food intake variation between phases was not significant in the control plot ($F_{2,42} = 0.905$, $p = 0.413$), or in the low ($F_{2,30} = 1.775$, $p = 0.189$) and medium-concentration plots ($F_{2,36} = 2.342$, $p = 0.112$). However, food intake significantly varied among phases in the highest concentration plot ($F_{2,61} = 3.529$, $p = 0.036$), and results showed a significant increase in food intake between control and experimental phases (Tukey's HSD, $p = 0.028$), but no differences were found between control and post-treatment phases (Tukey's HSD, $p = 0.599$) or between experimental and post-treatment phases (Tukey's HSD, $p = 0.599$) (Fig. 3). For sex, females (0.187 ± 0.007) showed significantly higher food intake than males (0.149 ± 0.010). Furthermore, breeding condition also explained variation found in food intake, because non-breeding individuals (0.189 ± 0.008) showed higher food intake than breeding individuals (0.131 ± 0.007). Regarding the interaction between phase × recapture, food intake did not vary between new and recaptured individuals during the control (*T* test, $p = 0.470$) or post-treatment phases (*T* test, $p = 0.341$). However, captured individuals significantly reduced food intake during the experimental phase compared with new individuals (*T* test, $p = 0.014$) (Fig. 4).

Discussion

Scent marks left by potential predators have previously been documented to trigger antipredatory responses for many rodents, such as *Lasiopodomys brandtii* (Hegab et al. 2014), *Microtus agrestis* (Dickman and Doncaster 1984; Bolbroe et al. 2000), *Clethrionomys glareolus* (Dickman and Doncaster 1984), and *A. sylvaticus* (Dickman and Doncaster 1984; Navarro-Castilla and Barja 2014a, b). However, this is the first study to demonstrate that wood mice are able to distinguish between different concentrations of predator faeces and responding accordingly.

Antipredatory behaviour cannot persist indefinitely because of the associated costs (Preisser et al. 2005), so

Table 1 General linear model results regarding the effect of phase, sex, breeding condition, recapture, and interactions on the amount of food intake in wood mice under different degrees of predation risk

Factor	*F* value	*df*	*p* value
(Intercept)	853.730	1	0.0001
Phase	8.044	2	0.0001
Sex	9.665	1	0.002
Breeding condition	17.614	1	0.0001
Recapture	0.007	1	0.935
Phase × plot	2.107	9	0.030
Phase × recapture	3.052	2	0.049

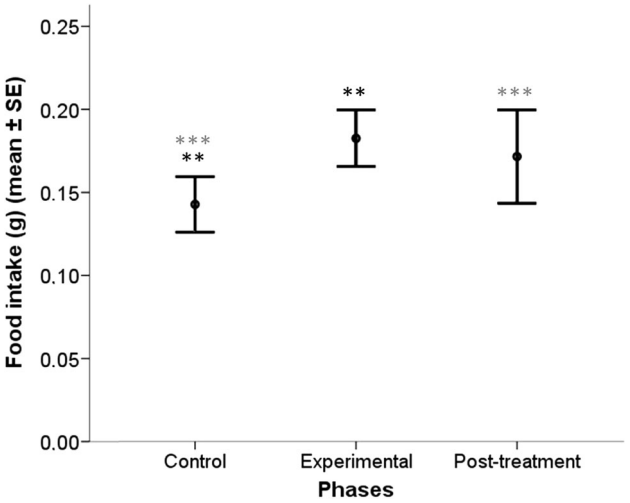


Fig. 2 Mean food intake by wood mice during control, experimental, and post-treatment phases. *Asterisks* indicate significant differences between the analysed groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

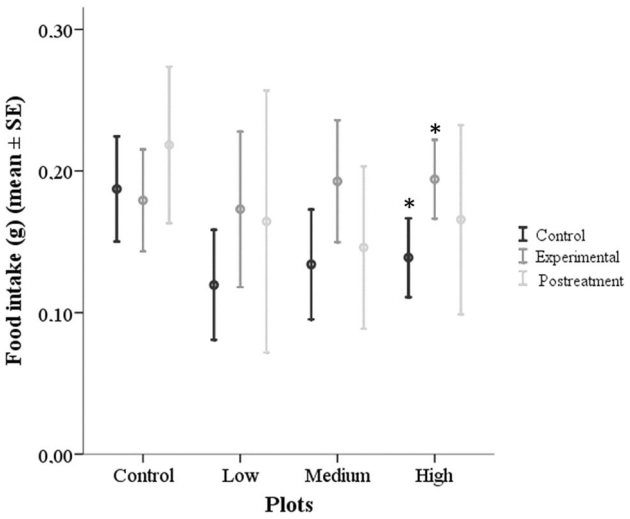


Fig. 3 Mean food intake by wood mice in each plot during the three phases. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

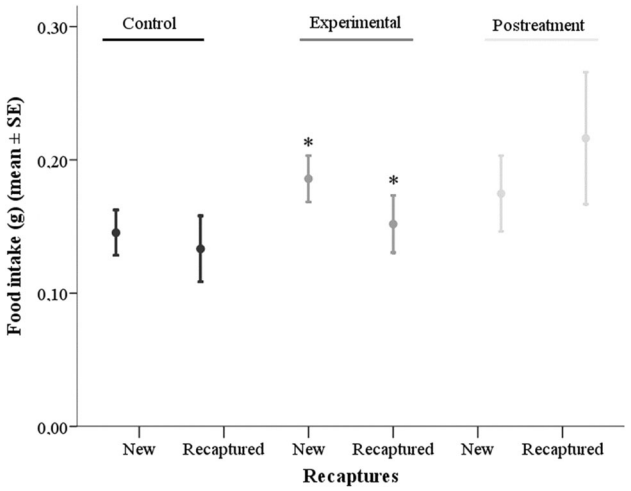


Fig. 4 Mean food intake by wood mice in the different phases relative to the recapture factor. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

prey have to balance the distribution of their daily and antipredatory activities suitably throughout the day (Lima and Bednekoff 1999; Sih and McCarthy 2002; Mirza et al. 2006). The present study showed that individuals modified a daily non-defensive routine, food intake, based on perceived predation risk. Thus, the amount of food ingested during the experiments was determined by phase and plot. In the control plot, there was no variation in food intake between the control and experimental phases; however, we found that food intake significantly positively increased with increasing red fox faecal concentration in the highest concentration plot. Thus, food intake accordingly increased as the perceived predation risk was higher in the different plots, which showed an interesting and important trend that should be studied more in depth in future projects. The predation risk allocation hypothesis (Lima and Bednekoff 1999) predicts that an animal under long and frequent high-risk periods should allocate more feeding effort over short low-risk intervals. However, the previous experiments on feeding behaviour under predation risk have yielded both reductions and increases in food intake near the scents of a potential predator in many prey organisms, such as *Aplodontia rufa* (Epple et al. 1993), *Arvicola terrestris* (Barreto and Macdonald 1999), *Oryctolagus cuniculus* (Boag and Mlotkiewicz 1994), and *A. sylvaticus* (Navarro-Castilla and Barja 2014a). In our study, during the experimental phase and especially in the high-concentration plot, individuals had to trade-off security and vital necessities in response to the previously perceived degree of predation risk before going into the trap and while being captured, because red fox faecal odour is likely also perceived inside the trap. If we consider traps to be shelters, we suspect that the possible safety effect of the traps would be more important for mice that experience a higher degree of

predation risk. Because the risk allocation hypothesis proposes that animals, during high-risk periods, should allocate more feeding effort in low-risk intervals, the sensation of safety inside traps for mice would be greater when the perceived risk also increases, and this could explain the increased food intake found under the highest predation risk situation compared with the other treatments and control. Similar results after stressful events have been previously observed, and animals increased food intake after being subjected to stressful situations (Wilson and Cantor 1986; Hamilton and Heithaus 2001).

In nature, the risk of encountering a potential predator decreases as time passes, especially in the case of predators that have extensive foraging areas, such as the red fox (Kats and Dill 1998). Thus, we expected that the degradation of volatile compounds simulated in the post-treatment phase of this present study could have been interpreted by prey as a reduction in the probability of being caught by a predator (i.e., a shorter and infrequent high-risk situation rendered it unnecessary to maintain antipredatory behaviour (Lima and Bednekoff 1999). In relation to this, Hegab et al. (2014) recently demonstrated that faeces stored for longer periods lead to a reduction of perceived predation risk, because prey decreased antipredatory responses under laboratory conditions. In our study, increased food intake observed in the experimental phase remained during the post-treatment phase instead of showing a significant reduction. This unexpected result could have been obtained, because faecal concentrations 5 days after deposition might not be low enough to significantly reduce prey response. In addition, we found noticeably greater variation during the post-treatment phase than the other phases, which could be due to reduction in the number of individuals captured in this phase (control phase $n = 80$; experimental phase $n = 57$; and post-treatment phase $n = 32$), dilution effects on the four different plots, and different reactions caused by individual characteristics (e.g., sex and breeding condition). Nevertheless, concentration of volatile compounds seems to be an important factor in the assessment of predation risk, but further studies are needed to fully understand the antipredatory responses of prey under natural conditions.

The amount of food intake was also affected by breeding condition, because non-breeding individuals showed higher food intake. Although energy requirements are higher during breeding season (Speakman 2008; Dantzer et al. 2010), breeders have also to take care of their litter and avoid taking risks and prioritizing breeding and mating (Montgomery et al. 1991). However, females also showed higher intake, which could be related probably to greater dependence on resources than males (Montgomery et al. 1991; Penn and Smith 2007). Finally, recapture also had an effect on food intake. Recaptured individuals may have reduced their food intake during the experimental phase in response to a second encounter with predator's odour because of the previous non-harmful experience. Thus, when wood mice came across predator's faecal marks again, the perceived risk might have been lower, because in the preceding catch, they were able to obtain food easily without suffering risk (i.e., no predator attack). As a result of previous experience, they seemed to learn that the chemical signals would not be as dangerous as they thought the first time. Furthermore, experience is often treated from a coevolutionary aspect by scientists, without considering the mouse life history perspective. Therefore, although the previous predator–prey studies have produced mixed results (Dickman 1992; Monclús et al. 2005; McEvoy et al. 2008; Navarro-Castilla and Barja 2014a), it is clear that experience has a role in shaping prey behaviour, and future studies should take this into account to produce more comprehensive results.

In conclusion, red fox faeces seem to be an important cue for wood mice which are able to evaluate different degrees of predation risk based on altering food intake. Thus, wood mice increased food intake as a behavioural strategy in response to long-frequent high-risk situations, and consistently did so over time even when the threat was gone. In addition, our results suggested that this antipredatory response seems to be modulated by individual factors, such as sex, breeding condition, and life experience. Consequently, prey behavioural responses to predation risk cannot be generalised, and further research is necessary to elucidate how prey species cope with each risky situation.

Acknowledgements The authors wish to thank the Comunidad Autónoma of Madrid (Spain) for providing the permits required to conduct this study and the Universidad Autónoma de Madrid (Spain) for allowing this project. We are grateful to Cañada Real Open Centre, especially to José España, for supplying the red fox faecal material needed to carry out the experiments. We would also like to thank Niamh Walsh for her English revision of the manuscript.

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